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COUPLED Na^+ - K^+ EXCHANGE IN THE RABBIT DETRUSOR MUSCLE

BY

JULIE LYNN MUNSON

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Coupled Na^+ - K^+ Exchange in the Rabbit Detrusor Muscle" submitted by Julie L. Munson in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The volume of the extracellular space of the rabbit detrusor muscle was studied using [^{14}C]-labelled mannitol, sucrose, inulin and dextran. Two distinctly different distribution values were reached; mannitol and sucrose equilibrated at about 60 ml/100 g, while inulin and dextran equilibrated at about 45 ml/100 g. The equilibration values of inulin and mannitol were not significantly altered by incubation of tissues in the presence of ouabain, but incubation of tissues in the presence of metabolic inhibitors greatly increased the equilibration values of these two compounds. It was concluded that too many variables existed in order to adequately estimate the volume of the extracellular space and thus determine the intracellular concentration of ions.

The ability of Na^+-rich rabbit detrusor muscle to extrude Na^+ in the presence of K^+ was studied by following net ion movements. Na^+ extrusion in exchange for K^+ was temperature sensitive and could be inhibited by the cardiac glycoside, ouabain. Na^+ extrusion was inhibited by the combination of anoxia and omission of D-glucose from the media, but not by either procedure alone. Under anaerobic conditions, D-glucose and D-mannose were the only substrates capable of supporting Na^+ pumping. DNP demonstrated a preferential inhibition of K^+ accumulation at 1 mM, provided that a suitable glycolytic substrate was present in the medium. Omission of a substrate in the presence of DNP resulted in total inhibition of ion pumping. Similarly, the partial inhibition of ion recovery produced by 10 mM azide was rendered complete upon the omission of D-glucose from the media. 2DG proved incapable of inhibiting glycolysis under aerobic conditions, but successfully competed with glucose and inhibited ion recovery under anaerobic cond-

itions. Both NEM and IAA completely inhibited ion recovery at a concentration of 1 mM, and caused tissue swelling. Addition of pyruvate to the media was unable to reverse the inhibitory effects of NEM, but pyruvate, lactate, β-hydroxybutyrate and oxaloacetate were able to reverse the effects of IAA under aerobic conditions.

The present study has shown that coupled Na^+-K^+ pumping in the rabbit detrusor muscle is similar to Na^+ pumping mechanisms in other smooth muscles in that it is temperature sensitive, inhibited by ouabain and metabolically dependent. Evidence has been presented to suggest that some other mechanism may also be involved in controlling the Na^+ and water contents of this tissue.

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[illegible]

LIST OF ABBREVIATIONS

IAA - Iodoacetic acid

DNP - 2, 4-dinitrophenol

NEM - N-ethylmaleimide

2DG - 2-deoxyglucose

ATP - Adenosine triphosphate

I. INTRODUCTION

INTRODUCTION

The ability of the cell to maintain an internal solution vastly different in molecular composition from the fluid normally bathing its exterior surfaces has stimulated many scientific investigations. The cell membrane is the only barrier between the two differing solutions and has consequently been attributed with possessing a number of functions vitally important to the integrity of the cell. Any passage of material into or out of the cell must go through this membrane, whether by simple diffusion or by a selective process of the membrane itself.

Wilbrandt first introduced the term active transport in 1937 to describe a process whereby a substance is moved against its electrochemical gradient at the expense of energy produced by the metabolic processes of the cell. Two main theories have been advanced to explain the source of this energy. The redox-pump theory (Conway; 1951, 1953, 1955) postulated that the energy for active ion transport was obtained by coupling a series of electron transfers down a chain of carriers to the final carrier, oxygen. Caldwell (1960) found that the injection of ATP, or compounds which could generate ATP, into poisoned squid giant axons led to an efflux of Na^+, suggesting that energy-rich phosphate compounds were involved in the mechanism of Na^+-extrusion. Evidence accumulated to indicate that any process which interfered with the production of ATP, or increased its breakdown, also interfered with active transport. Thus ATP was thought to be the direct energy source for active transport, (Hoffman, 1960; Whittam, 1962); energy was released by its hydrolysis and ATP was subsequently resynthesized from creatine phosphate and ADP under the influence of the enzyme creatine

kinase (Lohmann reaction). There is, however, still the possibility that other high energy phosphates may be involved in supplying energy for ion transport, a theory supported by several workers (Hill and Morales, 1951; Slater, 1953).

The normally high intracellular potassium and low intracellular sodium concentrations present in muscle fibres were first thought to be due to the fibres being impermeable to sodium. Mond and Netter (1930, 1932) proposed that the cell membrane was impermeable to sodium and chloride ions, but freely permeable to potassium ions which were exchanged for hydrogen ions. Conway and Boyle (1939) believed that the electrical equilibrium across the cell membrane demanded a Donnan equilibrium, so that the product of the cations and anions (including impermeable anions) within the cell would be equal to that outside the cell. Consequently they proposed that the muscle fibre was permeable to chloride and other anions, but not to sodium, and that potassium fluxes must be accompanied by anion fluxes in order to preserve electroneutrality. However, Fenn and Cobb (1934) demonstrated that muscles could accumulate sodium when immersed in a potassium-free medium and this was subsequently confirmed by Heppel and Schmidt (1938) and by Steinbach (1940). It thus became apparent that the cell membrane of muscle was indeed permeable to sodium; Heppel and Schmidt, in fact, went as far as to suggest the possible existence of a membrane pump which excreted sodium from the cell. Fenn (1936) suggested that the sodium accumulated was exchanged for intracellular potassium. Dean (see review, 1941) was the first to argue definitively in favor of a sodium pump, demonstrating that it was the only theory which could explain the exchange of sodium for potassium on the assumption that the cell was permeable to sodium. With such

a pump the cell would be continuously excreting sodium, in exchange for potassium, instead of only pumping out the small amounts of sodium which had leaked in.

In a review of membrane ion transport, Ussing (1949) concluded that there was little evidence for an active transport of potassium in muscle or nerve, and that the high internal potassium content of cells was due to two factors; namely, the Donnan equilibrium and the binding of potassium to intracellular proteins. Ussing, and Conway and Hingerty (1948) explained the movement of potassium as exchange diffusion. In 1950 Flynn and Maizels demonstrated an active sodium extrusion mechanism in human erythrocytes, giving evidence which they felt indicated that potassium movements were purely passive. Conway (see review, 1957) claims to be the first to have unequivocally demonstrated an active sodium extrusion mechanism in muscle fibres.

In 1952, Steinbach demonstrated a linked sodium-potassium active transport mechanism in frog muscle. Hodgkin and Keynes (1953) provided evidence for a similar linked pump in nerve. Similarly Harris (1954) and Maizels (1954) were able to demonstrate a coupling of the active movements of sodium and potassium in human erythrocytes. Harris stated "...it is assumed that an active process potentiated by metabolic reactions causes an outward flux of Na ions and an inward flux of K ions so linked that the inward active K flux is numerically related to the outward active Na flux".

The actual transport mechanism of sodium and potassium ions was postulated to be a carrier complex located within the membrane which had a specific site for sodium on the inside of the membrane and a specific site for potassium on the outside of the membrane. Ussing (1948) hypo-

thesized that either the attachment of the ions to the carrier molecule or the subsequent separation of the ions from the carrier complex would require coupling to an energy source. Skou (1965) outlined the requirements for a system transporting sodium. In addition to a membrane location and specifically orientated sites for sodium and potassium, Skou stated that an enzyme, capable of hydrolyzing ATP and converting this energy into cation movement, would have to be contained in this complex. The rate of ATP hydrolysis would have to be dependent upon the internal sodium and the external potassium concentrations. This system would have to be common to all cells which carried out an active linked transport of sodium and potassium.

In 1957 Skou demonstrated the presence of an enzyme in the leg nerve of the crab which caused the hydrolysis of ATP to ADP. This ATPase required magnesium ions to function and was maximally activated in the presence of sodium and potassium. Higher concentrations of potassium, however, inhibited the activity of the enzyme stimulated by sodium. Calcium ions completely inhibited the ATPase activity, although this inhibition could be overcome by increasing the magnesium concentration. Cardiac glycosides also inhibited ATPase activity. Skou therefore suggested that this enzyme required Na^{+}-Mg^{++}-ATP as a substrate.

Evidence soon accumulated that ATPases were present in a variety of tissues, all of which could be inhibited by cardiac glycosides such as ouabain, which also inhibited the active transport of sodium. Glynn (1962) demonstrated that the glycoside-sensitive ATPase of human erythrocytes was activated maximally when sodium was inside the cell and potassium was outside the cell. He found that for each inorganic phosphate ion formed,

three sodium ions were extruded, but that less than three potassium ions were taken up; thus it was not a one to one exchange. Whittam (1962) reported similar findings using erythrocyte ghosts. By loading the ghosts with ATP, and varying the internal and external concentrations of sodium and potassium he was able to show that ATP hydrolysis within the ghosts was maximal with potassium in the medium and sodium within the ghosts. He proposed that active sodium efflux was directly proportional to the internal sodium concentration and dependent on external potassium ions, and that the potassium influx was coupled to the sodium efflux. Caldwell and Keynes (1959) demonstrated in squid giant axons that inhibition of sodium extrusion by cardiac glycosides occurred only if the glycoside was applied to the exterior of the cell. In red cell ghosts it was also shown that ATP was required inside the cell, that potassium was required on the outside of the membrane, that magnesium and sodium were required on the inside of the membrane, and that calcium ions inhibited the splitting of ATP on the inside of the membrane (see reviews by Glynn, 1964; and Skou, 1965).

This enzyme became known as the (Na^+ + K^+)-activated ATPase and demonstrated many of the properties associated with the "carrier" which is believed to be involved in the transport of both Na^+ and K^+ across cellular membranes (see reviews by Charnock and Opit, 1968; and Dunham and Gunn, 1972). It has been found in a variety of cell types and has been shown to be an integral part of the cell membrane, the hydrolysis of ATP by this enzyme requires the presence of both Na^+ and K^+ and is believed to occur by a reaction sequence involving a phosphoprotein intermediate complex:

1. $\text{Enzyme} + \text{ATP} \xrightarrow{Na^+} E_1{\sim}P + \text{ADP}$
2. $E_1{\sim}P \longrightarrow E_2{\sim}P$

$$3.\quad E_2{\sim}P + H_2O \xrightarrow{K^+} \text{Enzyme} + P$$

Sodium is an absolute requirement for the operation of this enzyme and cannot be replaced by any other cation. Potassium can be replaced to varying degrees by other cations. The rate of ATP hydrolysis can be very sensitive to small changes in the concentration of either of the coions involved, the optimal ratio of $Na^+:K^+$ varying under different experimental conditions. In all cases Mg^{++} is required for maximal activation of the enzyme in combination with Na^+ and K^+. As with active transport this enzyme is inhibited by low concentrations of cardiac glycosides, which are believed to act at step 3, and by oligomycin which is believed to inhibit the enzyme conversion at step 2. In intact structures such as the red cell ghost, this enzyme has been shown to possess vectorial properties, Na^+ being required on the interior of the cell and K^+ activation and ouabain inhibition occurring on the outside of the cell.

Several models have been proposed for the mechanism by which Na^+ and K^+ are transported across the cell membrane, but experimental proof of any one model has not yet been obtained. Any model proposed must account for the various properties of ion transport already described; that is, vectorial properties, coupling of ion movements to some form of energy production, inhibition by cardiac glycosides as well as many metabolic inhibitors. The various molecular carrier models for ion transport have been forsaken in lieu of a conformational change model involving the $(Na^+ + K^+)$-activated ATPase. One such model has been proposed by Opit and Charnock (1965) in which the ATPase, as a part of the protein structure of the membrane, rotates about the central lipid core as a result of the reaction sequence involved in the dephosphorylation of ATP. Similar conformational

change models have been described by Albers (1968), Hokin (1969), Robinson (1970). Proof of the validity of these models, as well as many others, awaits the isolation and identification of the proposed intermediate complex.

Effect of Metabolic Inhibitors on Transport of Electrolytes

A wide variety of metabolic inhibitors have been used in studies of the metabolic requirements of active transport in different tissues. In 1941 Danowski demonstrated that glucose was essential for the active movement of cations in erythrocytes, and that the addition of fluoride was capable of inhibiting these movements. Maizels (1949) confirmed these results when he showed that sodium-rich erythrocytes incubated in glucose-free media were unable to extrude sodium or accumulate potassium. Maizels also varied the pH to indicate that at pH's which inhibited glycolysis the movement of cations was also inhibited. In 1951 Maizels tested the effects of metabolic inhibitors and of glucose substitutes on glycolysis and active cation transport in human erythrocytes. Fluoride and iodoacetic acid both inhibited glycolysis and active sodium extrusion. Cyanide, azide, and carbon dioxide (oxidative inhibitors) were unable to prevent the movements of sodium and potassium. Dinitrophenol (DNP), malonate, arsenate and mepacrine also produced no effects on the cation transport. D-glucose, D-mannose, and D-fructose were able to support active transport, but D-galactose was not, presumably because of a lack of affinity of this sugar for the enzyme hexokinase. Arabinose, xylose, maltose and lactose were able to support the extrusion of small amounts of sodium. Pentoses appar-

ently enter the red cell rapidly, whereas disaccharides like sucrose do not, and are thus unable to maintain active transport. Pyruvate and lactate were unable to support active transport, because active transport in erythrocytes is energized solely by the glycolytic pathway.

Similar results were obtained in skeletal muscle by Shaw and Simon (1955), who found that sodium extrusion was inhibited by iodoacetic acid, but not by azide, cyanide or DNP. In contrast, complete inhibition of sodium extrusion and potassium accumulation was produced by 0.1 mM DNP in squid giant axons (Hodgkin and Keynes, 1955). Similar results were produced by cyanide and azide and by cooling to 1°C. All these effects were reversible upon washing out the inhibitor or raising the temperature. Carey, Conway and Kernan (1959) showed that both cyanide and anoxia inhibited sodium extrusion by about 60% in frog sartorious muscle, while 2 mM iodoacetate completely inhibited sodium extrusion. Net extrusion of sodium was also completely inhibited at 0°C. DNP, however, caused an increase in sodium extrusion, possibly because of the stimulation of glycolysis produced by DNP, despite the inhibition of oxidative phosphorylation reflected in a decrease in the amount of ATP in the muscle.

The effects of metabolic inhibitors on sodium pumping in nerves and skeletal muscle have been extensively reviewed by Shanes (1958) and Conway (1957) respectively.

Sodium Pumping in Smooth Muscle

Smooth muscles have been shown, in general, to have a higher sodium and chloride content and lower potassium content than skeletal muscle (Daniel, 1958; Burnstock *et al.*, 1963). These findings have raised the question as to whether the mechanism of maintenance of ion gradients in smooth muscle was different from that in striated and cardiac tissue. Daniel (1958) studied the electrolyte contents of cat, rabbit and human myometrium, and of guinea-pig and rabbit taenia coli. On exposure to Krebs-Ringer solution, all the tissues lost potassium and gained sodium; the loss of potassium approximately equaled the gain of sodium in all tissues except the human myometrium. Daniel suggested that potassium was not distributed according to its equilibrium potential, and the evidence presented suggested an active transport of this ion. Sodium was also postulated to be actively extruded from smooth muscle. Further work by Daniel and Robinson (1960a) confirmed these hypotheses. These authors demonstrated an active sodium extrusion linked to potassium uptake in uteri from rabbits and cats. Tissues exposed to potassium-free Krebs-Ringer at 4°C showed a loss of potassium and an equivalent gain of sodium, which was reversed upon exposure to a potassium containing media at 37°C. Incubation in potassium-free media at 37°C prevented the active extrusion of sodium and thus they concluded that the most probable mechanism of ion transport was an active sodium extrusion linked to an uptake of potassium. Dawkins and Bohr (1960) found in rat aorta that the chemical composition changed drastically when tissues were placed in a physiological salt solution after dissection. Sodium was lost and potassium was gained. The gradients gradually recovered, how-

ever, although the steady-state which they eventually reached was not the same as that in vivo. Goodford and Hermansen (1961) reported a similar situation in guinea-pig taenia coli. After dissection and immersion in a physiological salt solution, the tissues rapidly lost sodium and gained an equivalent amount of potassium until a steady-state was reached which never equaled that of control tissues. Kao and Siegman (1963) investigated electrolytic exchange in rabbit myometrium. More potassium was present intracellularly than could be accounted for on a strictly passive basis. Potassium and sodium fluxes were studied and it was found that both net fluxes were uphill and equal but in opposite directions. No net extrusion of sodium occurred in a potassium-free medium and the ^{22}Na efflux was reduced when potassium was removed from the external medium. The authors stated that the sodium pump was probably coupled to a potassium pump in this tissue.

The sodium pump in smooth muscle was shown to be affected by metabolic inhibitors in a similar fashion to nerve and skeletal muscle. Daniel and Robinson (1960b) used sodium-rich cat and rabbit uteri to demonstrate that the inhibitors of glycolysis, IAA and fluoride, inhibited active transport whereas anaerobiosis and oxidative inhibitors (cyanide, azide, antimycin and malonate) had little effect. Ion transport was completely inhibited by ouabain. 2.5 mM DNP inhibited potassium accumulation but had less effect on sodium extrusion. Reducing the temperature from 37°C to 24°C had little effect on the transport processes but further reduction to 4°C completely inhibited active transport. Freeman-Narrod and Goodford (1962) examined the movements of sodium and potassium in guinea-pig taenia coli at different temperatures. They found that the ionic

composition of the tissue reached a steady-state condition more rapidly at 35°C than at 20°C or 4°C. The critical dependence of sodium movement on temperature suggested that the transport process was dependent upon a supply of metabolic energy.

Recently a large amount of evidence has accumulated to suggest that the intracellular ion contents and volume of the cell may be controlled by mechanisms other than the coupled Na^+-K^+ pump described above. Following work by Kleinzeller and Knotkova (1964), a second pump was described for kidney slices by Macknight (1968), and Whittembury and Proverbio (1968). This pump was dependent on external Na^+ but not on external K^+, was insensitive to ouabain but was temperature sensitive and could be inhibited by ethacrynic acid. Garrahan and Glynn (1967a) demonstrated a Na^+:Na^+ exchange in erythrocytes incubated in K^+-free solution. This exchange was inhibited in the presence of K^+ and by ouabain, and was believed to represent an exchange of intracellular Na^+ for extracellular Na^+ via the Na^+-K^+ pump mechanism (Sachs, 1970). An ouabain sensitive Na^+ outflux occurring in Na^+-free solutions has also been demonstrated by Garrahan and Glynn (1967a,b).

In smooth muscle, Friedman and his coworkers (1968) found that Na^+ was extruded from the rat tail artery in Na^+-free medium but this was not accompanied by an uptake of K^+. This Na^+-extrusion mechanism was temperature sensitive. Daniel (1967) has shown that ethacrynic acid inhibited Na^+-extrusion in rabbit uterus, but had no effect on the K^+ accumulation in this tissue. In 1969, Daniel et al. found that Na^+ exchange across the cellular membranes of rat uterus consisted of two components - an ouabain-sensitive component and an ouabain-insensitive, but ATP-dependent component.

Further work on rabbit aorta and rat uterus (Daniel and Wolowyk, 1971; Daniel and Robinson, 1971a,b) showed that ouabain and K^+-free solutions inhibited both ion movements and water movements. They suggested that in smooth muscle there exists an ouabain sensitive transport of Na^+ which controls Na^+ and K^+ contents of the tissue, and a metabolically-dependent ouabain-insensitive Na^+ transport which controls cell volume. No evidence was found, however, of an exchange diffusion of sodium via a sodium selective carrier (Daniel and Robinson, 1971a,b).

Methods of Study of Ion Gradients

To determine the relationship between ion movements and excitability of smooth muscle cells, flux studies of the various ions have been examined, usually with the aid of radioisotopes. However, it is necessary to know the intracellular concentration of the ions in order to study their movements through the membrane. This has generally been done by determining the net amount of the ion present in the tissue and subtracting from it the amount of ion present in the extracellular fluid. This has required an accurate measurement of the extracellular space in the tissue, which has posed many problems (see Goodford, 1968). To determine the amount of extracellular space in a tissue, a molecule is required which does not penetrate the cell membrane, is not osmotically active, is not metabolized or bound, yet is small enough to diffuse completely throughout the extracellular fluid.

In smooth muscle the size of the extracellular space varied depending upon the marker used. Thus Daniel (1958) found that the extra-

cellular space in uterine tissues varied in size from 400-700 ml/kg with chloride, Na^+ or sulphate. Bozler (1961) studied the distribution of non-electrolytes in the frog stomach muscle and sartorius, obtaining widely varying results with substances such as glycerol, urea, thiourea, erythritol, arabinose, sucrose and inulin. He concluded that some sugars and electrolytes penetrated into the muscle fibres. Goodford and Hermansen (1961) estimated the extracellular space of the guinea-pig taenia coli using radioactive inulin, lithium, sodium and polyglucose, as well as by electrom microscopy. They concluded that polyglucose was too large an ion to penetrate throughout the extracellular space whereas inulin could get into places that polyglucose could not. Values ranged from 20% with electron microscopy to 70% with ^{24}Na. Ogston and Phelps (1961) suggested that an even larger space could be obtained with inulin if it were not for the steric hindrance of large molecules such as hyaluronic acid in the extracellular space. To test this, Goodford and Leach (1964) incubated their tissues with hyaluronidase and found that the extracellular space measured with inulin increased slightly. In 1965, Barr and Malvin estimated the extracellular space of canine intestinal smooth muscle, using a number of different-sized molecules: urea, arabinose, mannitol, sucrose,raffinose, inulin, and radioiodinated serum albumin. They found that the size of the extracellular space increased with a decrease in the molecular size of the marker, and concluded that intracellular ion concentrations could best be calculated if the extracellular space value was between that of sucrose and RISA. Values for the extracellular space of a wide variety of smooth muscles are reviewed by Burnstock _et al_. (1963), Goodford (1968), and Burnstock (1970). However, no values are quoted for the detrusor muscle

of any species.

An incorrect estimate of the size of the extracellular space will lead to incorrect values for the intracellular concentration of ions. The division of the net contents of ions in the tissue into two compartments, extracellular and intracellular, is based on the assumption that the ions in both compartments are dissolved in the total tissue water and are freely exchangeable. However, a great deal of evidence has accumulated to indicate that Na^+, in particular, is multicompartmental in nature (Goodford, 1962; Daniel and Robinson, 1970; Jones, 1970). Na^+ may be bound in the extracellular space (Headings et al., 1960; Palaty et al., 1969; Jones and Karreman, 1969) as well as intracellularly (Jones, 1970). Because of the problems involved in accurately determining the intracellular ion concentrations, and consequently their unidirectional fluxes, it is often easier to study the net ion movements of tissues. Net ion movements are enhanced if the tissue is incubated in K^+-free media at 4°C, whereby the metabolic functions of the cell are inhibited and the ions run down their concentration gradients to equilibrate with the media; that is, the tissues gain Na^+ and lose K^+. Restoration of these Na^+-rich tissues to a medium containing K^+ at 37°C will lead to an accumulation of K^+ and extrusion of Na^+ against their concentration gradients. That these movements represent active transport and not simply diffusion from the extracellular space in order to equilibrate with the media has been argued by Carey, Conway and Hingerty (1959) by the fact that the amount of Na^+ extruded under these conditions is far beyond that which would be expected due to simple diffusion. The addition of K^+ to Na^+-rich tissues will activate the Na^+ pump at a maximal rate and thus any inhibitory actions of drugs would be more

pronounced under these conditions.

The Aim of this Thesis

In this laboratory, Paton (1968) found that the contractility of the rabbit detrusor muscle was affected by many of the same conditions which affected ion transport in other smooth muscles. Osman and Paton (1971) demonstrated that transport of α-amino-isobutyric acid in the rabbit detrusor muscle exhibited many of the properties which are attributed to the sodium pump. Transport occurred against a concentration gradient, was temperature sensitive, and was decreased by metabolic inhibition. Ouabain inhibited the amino acid transport and transport was found to be markedly dependent on the presence of external sodium and potassium, and to some extent on magnesium. They suggested that amino acid transport in this tissue required the functioning of the sodium pump. Thus the purpose of this thesis was to attempt to characterize in more detail the sodium pump mechanism in this tissue.

Specifically, answers to the following questions were sought:

1. What marker can best be used to determine the volume of the extracellular space in this tissue?

2. What are the effects of metabolic inhibition on the extracellular space?

3. Is there evidence for an active sodium extrusion and potassium accumulation in this tissue?

4. If so, is the energy source for this transport process glycolytic or oxidative or both?

5. Under what conditions can this active transport process maintain the ionic gradients?

6. What substrates can be used as an energy source for active transport in this tissue?

II. METHODS AND MATERIALS

METHODS AND MATERIALS

Preparation of Tissues

Male New Zealand white rabbits weighing 2 - 3 kg were killed by a blow to the neck and their urinary bladders then quickly excised. After being dissected free of surrounding tissue, the detrusor muscle was opened longitudinally and the mucosa was carefully removed by dissection through the plain of cleavage between the mucosa and underlying muscle layers. The muscle was then dissected into the desired number of pieces, each weighing between 20 and 50 mg.

Tissues to be used for the determination of extracellular space were pre-incubated for 30 minutes at 37°C in Krebs solution, equilibrated with 95% oxygen and 5% carbon dioxide. Tissues to be used for ion determinations were made sodium-rich by immersion in potassium-free Krebs solution (wt:Vol = 5 g:4 l) at 4°C for 18 to 24 hours. The solution was changed at least once during this period.

Solutions

The Krebs solution used had the following composition (mM): NaCl, 116; KCl, 4.6; NaH_2PO_4, 1.2; $CaCl_2$, 1.5; $MgSO_4$, 1.2; $NaHCO_3$, 22; and D-glucose, 20. KCl was omitted without substitution in the potassium-free Krebs solution. Glucose-free solutions were substituted with 20 mM sucrose. In cases where different substrates were used as the sodium salts, the sodium concentration was maintained at 140 mM by omitting the appropriate amount of NaCl. All solutions were made using double-distilled water and pH was maintained at 7.3 - 7.5.

Solutions were equilibrated with either 95% oxygen and 5% carbon dioxide or 95% nitrogen and 5% carbon dioxide.

Determination of Extracellular Space

The following compounds were used in the determination of the extracellular space:

1. 1-[^{14}C]-D-mannitol, 16.8 mC/mmole, molecular weight 182, (Amersham/Searle Corp.).
2. U-[^{14}C]-sucrose, 10.4 mC/mM, mol. wt. 342, (Amersham/Searle Corp.).
3. Carboxyl-[^{14}C]-inulin, 1.45 - 3.65 mC/g, mol. wt. 5000 - 5500, (New England Nuclear Corp.).
4. Carboxyl-[^{14}C]-dextran, 4.63 mC/g, mol. wt.15,000 - 17,000, (New England Nuclear Corp.), referred to as light dextran.
5. Carboxyl-[^{14}C]-dextran, 0.87 mC/g, mol. wt. 60,000 - 90,000, (New England Nuclear Corp.), referred to as heavy dextran.

After pre-incubation, tissues were incubated for varying periods of time in 5 ml of Krebs solution containing the radioactive marker in tracer concentrations. Tissues were then removed, rapidly rinsed in Krebs solution, blotted, placed in pre-weighed scintillation vials and weighed. The tissues were then dissolved using NCS (Amersham/Searle), 1 ml/100 mg tissue weight, overnight at 37°C. Ten ml of Bray's Phosphor (Bray, 1960) was then added to each vial; the vials were cooled and dark-adapted. Duplicate 1.0 ml samples of media were counted with the tissue samples in a Picker Nuclear Liquid Scintillation Counter (Liquimat 110), having a counting efficiency of about

95%. Quenching was corrected for using the channels ratio technique. Extracellular space was expressed in ml/100 g wet weight and was calculated from the ratio:

$$\frac{\text{dpm/100 g wet weight of detrusor muscle}}{\text{dpm/ml incubating medium}}$$

Determination of Tissue Ion Contents

After tissues were removed from the potassium-free Krebs medium at 4°C, they were pre-incubated for 30 minutes in potassium-free Krebs at 37°C. Paired tissues were removed at this point and their ionic contents determined. The remaining tissues were transferred to the experimental media for varying periods of time. Paired control tissues were always kept in normal Krebs solution and analysed for ion content at the end of the incubation period. Unless otherwise stated, media was bubbled with 95% oxygen, 5% carbon dioxide. Upon removal from the incubation media, the tissues were blotted and weighed in pre-weighed test tubes to obtain the wet weight. They were then dried in an oven at 105°C for at least 24 hours, placed in a desiccator upon removal and allowed to cool to room temperature. The tissues were then weighed again to obtain the dry weight. Ions were extracted using one of the following methods:

Method I:

Tissues were dissolved in 0.1 ml concentrated nitric acid and 0.05 ml hydrogen peroxide, applied each day for three consecutive days and kept in a sandbath at 200°C. The residue was then dissolved in 25 ml of distilled water and the sodium and potassium contents

determined using an Eel flame photometer. Standard curves of sodium and of potassium were plotted for each experiment. For each series of samples duplicate blank values were taken of a test tube treated in the same manner as the tissues, and this blank value was subtracted from the sample value before being read off the standard curve. Total tissue water was calculated by subtracting the dry weight from the wet weight, multiplying by 1000 and dividing this by the wet weight, so as to express the water content as g/kg wet weight of tissue. Sodium and potassium contents are expressed as mmoles/kg dry weight.

Method II:

Dried tissues were incubated in 5 ml of 10% glacial acetic acid, 0.64% [illegible] HNO_3(Cotlove, 1958) overnight, then diluted to 25 ml with double distilled water and the ion contents determined by flame photometry in the same manner as described for method I. This second method was tested as to its efficiency in leaching out the ions, in comparison with method I, by dissolving the remaining tissue in concentrated nitric acid and hydrogen peroxide, as in method I, and determining the amount of sodium and potassium left in the tissue after the leaching process. Of twenty samples tested, the values for sodium and potassium were not significantly higher than normal blank values. It was thus concluded that method II was equally as efficient as method I for the determination of ion contents, and required less time for extraction.

Statistical Analysis

Results are expressed as the mean plus or minus the standard error of the mean. Statistical significance was determined using the Scheffe's Test for Multiple Comparisons (Edwards, 1968), with a probability level equal to or less than 0.05 considered to be significant.

III. RESULTS

RESULTS

I. Extracellular Space

A. Uptake of [^{14}C]-Labelled Compounds

In order to estimate the size of the extracellular space, the uptake of a number of [^{14}C]-labelled sugars, of varying molecular weight, was studied. These sugars were used because they are generally unable to penetrate the cell membrane to any marked extent and therefore the space which they occupy should be almost exclusively extracellular in location. The volume occupied by these tracers, measured as ml/100 g wet weight of tissue, was plotted against the duration of incubation in Figure 1. The uptake of [^{14}C]-inulin, (molecular weight of 5,000 - 5,500) gradually increased as a function of time, until at 2 hr equilibration was reached at a volume of about 45 ml/100 g. There was a significant difference between the distribution volumes at 1 hr and at 2 hr but the volumes at 2 hr and at 4 hr did not differ significantly.

The rate of uptake of light [^{14}C]-dextran (mol wt = 15,000 - 17,000) was very similar to that of inulin and in fact did not differ significantly from inulin at any time point. The rate of uptake of the heavier [^{14}C]-dextran (mol wt = 60,000 - 90,000) was much slower than any of the other [^{14}C]-compounds studied, and was only approaching an equilibrium after 4 hr, at which time the value was not significantly different from inulin or light-dextran.

The smaller molecular weight compounds, [^{14}C]-sucrose (mol wt = 342) and [^{14}C]-mannitol (mol wt = 182), demonstrated a very rapid uptake during the first 30 min, after which influx gradually slowed. At 2 hr

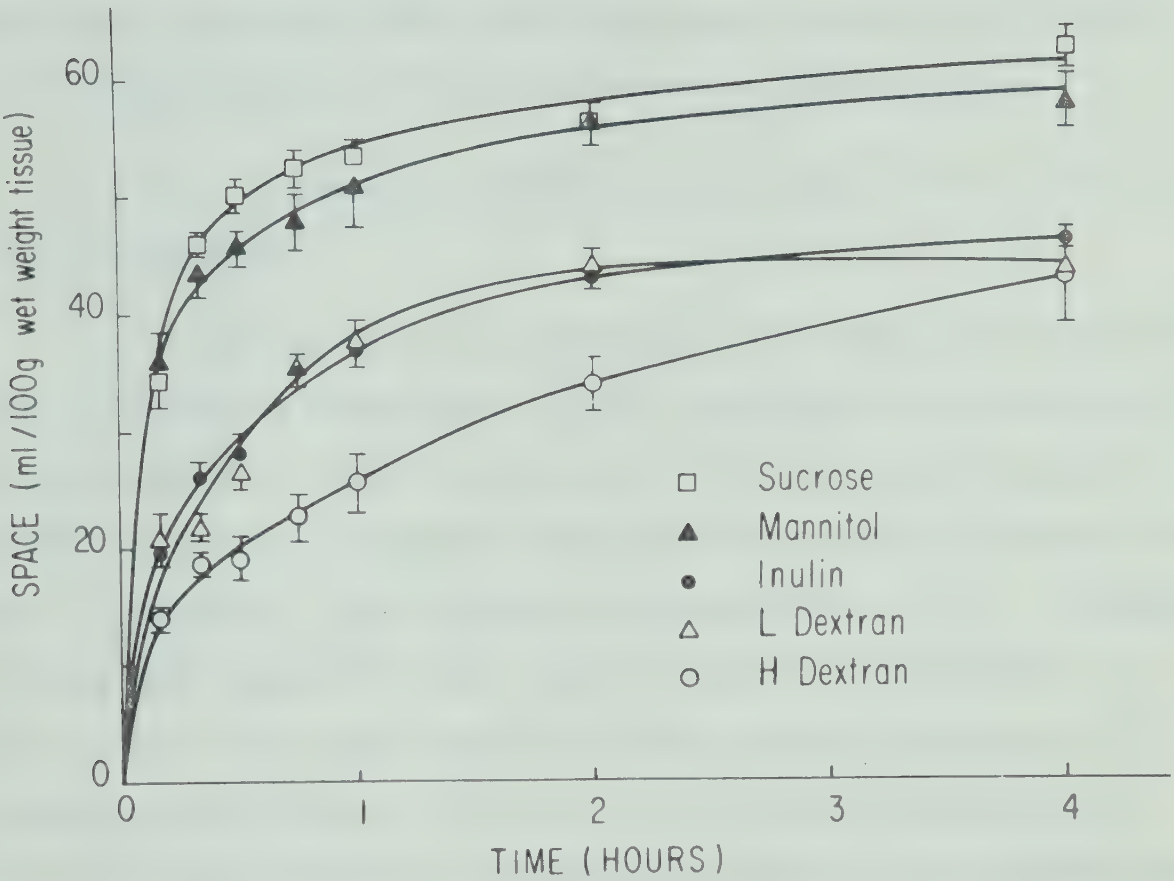

FIGURE 1. Uptake of extracellular markers by rabbit detrusor muscle. Tissues were incubated in normal Krebs solution containing tracer quantities of one of the following: [^{14}C] - mannitol, (▲); [^{14}C] - sucrose, (■); [^{14}C] - inulin, (●); [^{14}C] - light dextran, (△); or [^{14}C] - heavy dextran, (o). Each point is the mean ± S.E. of 6 observations.

the size of the space occupied by both [^{14}C]-sucrose and [^{14}C]-mannitol was about 56 ml/100 g increasing to about 60 ml/100 g after 4 hr. The values for mannitol and sucrose did not differ significantly at 4 hr, but were significantly larger than the volumes occupied by [^{14}C]-inulin, [^{14}C]-light dextran and [^{14}C]-heavy dextran at this time period.

B. Effect of Metabolic Inhibition on the Uptake of [^{14}C]-Inulin and [^{14}C]-Mannitol

The effects of various conditions on the uptake of [^{14}C]-inulin and [^{14}C]-mannitol were studied. First, the effect of the cardiac glycoside, ouabain, an inhibitor of the Na^+ - K^+ stimulated ATPase was examined. In previous experiments this agent had been shown to reverse the Na^+ and K^+ gradients of fresh tissues at a concentration of 10^{-4}M. Therefore tissues were incubated in Krebs solution containing 10^{-4}M ouabain for 30 min, then for an additional 4 hr in Krebs solution containing 10^{-4}M ouabain to which either [^{14}C]-inulin or [^{14}C]-mannitol had been added. In other tissues, metabolic inhibition was induced by pre-incubating the tissues for 40 min in glucose-free Krebs solution containing 1 mM of both 2,4-dinitrophenol (DNP) and iodoacetic acid (IAA), followed by 20 min incubation in glucose-free Krebs solution and a further 4 hr in glucose-free Krebs solution containing either [^{14}C]-inulin or [^{14}C]-mannitol. It can be seen (Figures 2 and 3) that ouabain had no significant effect on the volumes of distribution of either inulin or mannitol at 2 or 4 hr. However, exposure to IAA, DNP and glucose-free medium greatly increased the rate of influx as well as the 4 hr distribution volumes of both inulin and mannitol. After 4 hr, the inulin space had increased from the control value of 45 ml/100 g to about 65 ml/100 g while the mannitol space in-

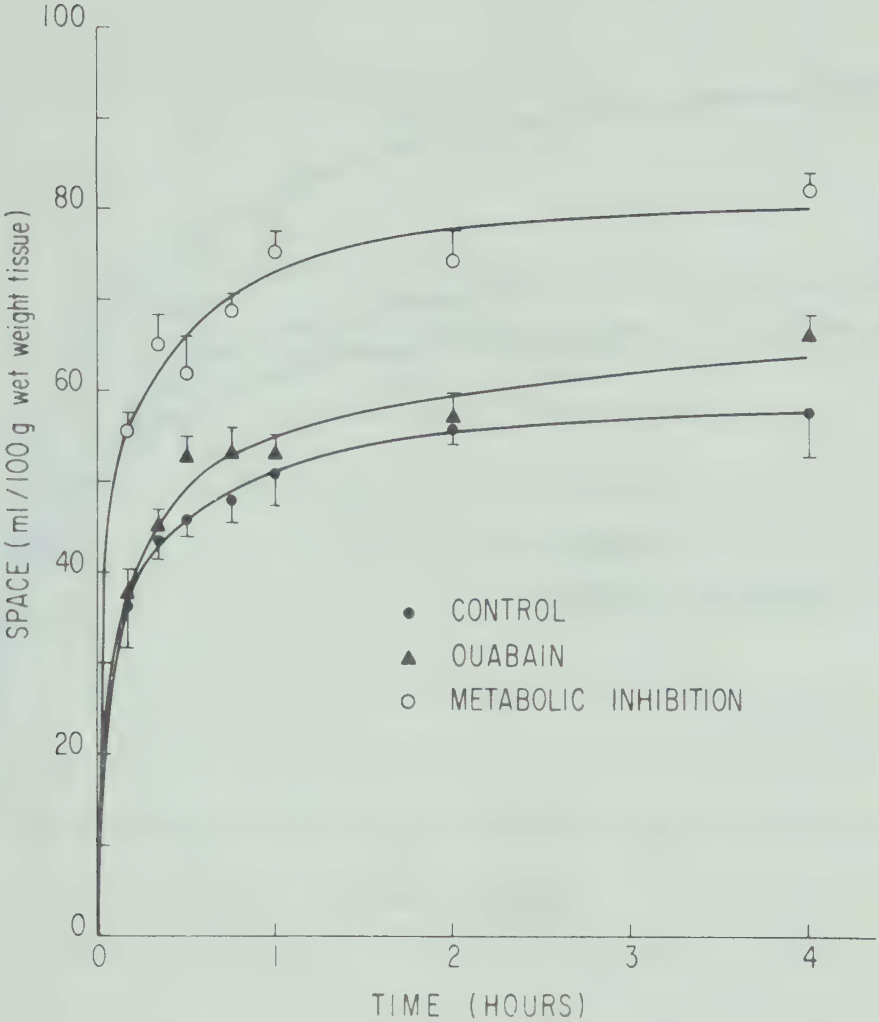

FIGURE 2. Effect of stress on the uptake of [^{14}C] - inulin by the rabbit detrusor muscle. Tissues were either pre-incubated in normal Krebs solution containing 10^{-4}M ouabain for 30 min., followed by an additional 4 hr. incubation in Krebs solution containing ouabain and [^{14}C] - inulin (▲); or tissues were incubated for 40 min. in glucose-free Krebs solution containing 1 mM DNP and 1 mM IAA, followed by 20 min. in glucose-free Krebs and another 4 hr. in glucose-free Krebs containing [^{14}C] - inulin (o). The uptake of the tracer is plotted against time and compared to the uptake of [^{14}C] - inulin in control tissues in the absence of any inhibitor (●). Each point represents the mean ± S.E. of 6 observations.

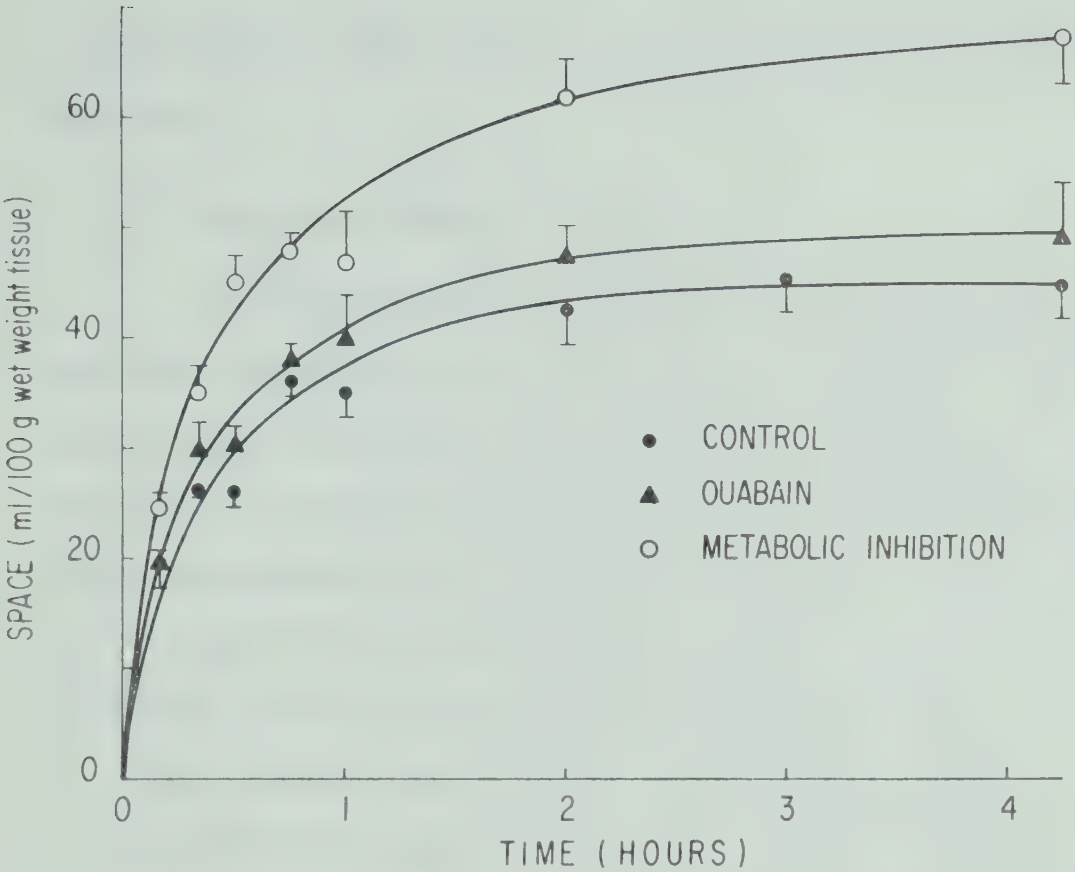

FIGURE 3. Effect of stress on the uptake of [^{14}C] - mannitol by the rabbit detrusor muscle. Tissues were either pre-incubated in normal Krebs solution containing 10^{-4}M ouabain for 30 min., followed by an additional 4 hr. incubation in Krebs solution containing ouabain and [^{14}C]-mannitol (▲); or tissues were incubated for 40 min. in glucose-free Krebs solution containing 1 mM DNP and 1 mM IAA, followed by 20 min. in glucose-free Krebs and another 4 hr. in glucose-free Krebs containing [^{14}C] - mannitol (o). The uptake of the tracer is plotted against time and compared to the uptake of [^{14}C] - mannitol in control tissues in the absence of any inhibitor (●). Each point is the mean ± S.E. of 6 observations.

creased from 60 ml/100 g to 80 ml/100 g in the presence of the metabolic inhibitors.

C. Electrolyte Contents of Tissues.

The tissue contents of Na^+ and K^+ as well as the total tissue water were determined after incubating tissues for 4 hr in Krebs solution and the values obtained are shown in Table 1. The intracellular contents of Na^+ and K^+ were then calculated using the different equilibration values obtained for the [^{14}C]-labelled compounds. It can be seen that the calculated intracellular concentration of Na^+ varied from -23.2 mM to 49.9 mM, and the intracellular K^+ concentration varied from 93.7 mM to 168 mM, depending upon the value used for the estimation of the volume of the extracellular space. The spaces occupied by inulin and the two dextrans were about 50% of the total tissue water and those occupied by mannitol and sucrose were about 70% of the total tissue water.

II. Net Movements of Sodium and Potassium

The ion contents of tissues freshly dissected and then allowed to recover ½ hr in Krebs solution, compared to the ion contents of tissues after Na^+-enrichment are shown in Table 2. After the usual 24 hr period of incubation in K^+ - free Krebs solution at 4°C, the tissues had gained approximately 330 mM/kg dry wt of Na^+ and lost about 290 mM/kg dry wt of K^+. These changes in ions were accompanied by a significant gain in the water content of the tissues, indicating that some degree of swelling has taken place. Rewarming the tissues at 37°C for ½ hr in K^+ - free Krebs solution (the period at which Na^+ - rich values were

TABLE I

DETERMINATION OF INTRACELLULAR Na^+ AND K^+ CONTENTS OF THE RABBIT DETRUSOR MUSCLE USING DIFFERENT VALUES FOR THE ECS

The calculations of intracellular Na^+ and K^+ contents were based on determinations of extracellular space and tissue H_2O, Na^+ and K^+ contents made after incubation in Krebs solution at 37°C for 240 min. Total tissue H_2O = 86.9 ml/100 g wet wt; total tissue Na^+ = 82.2 mmoles/kg wet wt; total tissue K^+ = 43.2 mmoles/kg wet wt.

EXTRACELLULAR MARKER	SPACE OCCUPIED (ml/100 g wet wt)	INTRACELLULAR CONTENT (mM)	
		Na^+	K^+
[^{14}C] - mannitol	58.2 ± 5.2 (5)	2.5	141.0
[^{14}C] - sucrose	62.9 ± 1.7 (4)	-23.2	168.0
[^{14}C] - inulin	44.8 ± 3.0 (6)	46.3	97.6
[^{14}C] - light-dextran	44.5 ± 2.1 (5)	44.6	97.2
[^{14}C] - heavy-dextran	43.0 ± 3.8 (6)	49.9	93.7

TABLE 2

EFFECT OF Na^+-ENRICHMENT ON ION CONTENTS

(a) Fresh tissues were incubated in Krebs solution at 37°C for 30 minutes after dissection.

(b) Na^+ - rich tissues were incubated in K^+ - free Krebs solution at 4°C for 18 - 24 hr.

(c) Tissues were rendered Na^+ - rich as in (b) and were then incubated for an additional ½ hr. in K^+ - free Krebs solution at 37°C.

(d) Tissues were rendered Na^+ - rich as in (b) and were then incubated for an additional 2½ hr. in K^+ - free Krebs solution at 37°C. Values are the mean ± S.E. of 48 - 52 observations.

* Indicates a value significantly different from the value for fresh tissues.

EXPERIMENTAL PROCEDURE	TISSUE CONTENT OF H_2O (g/kg wet wt)	Na^+ (mmoles/kg dry wt)	K^+ (mmoles/kg dry wt)
Fresh tissues[a]	844.4 ± 1.8	552.8 ± 11.0	321.0 ± 6.9
Na^+ - rich tissues[b]	856.8 ± 1.8*	880.8 ± 13.8*	33.2 ± 9.2*
Na^+ - rich tissues[c] exposed to K^+ - free solution at 37°C for ½ hr.	859.8 ± 3.0*	894.4 ± 16.9*	28.6 ± 1.7*
Na^+ - rich tissues[d] exposed to K^+ - free solution at 37°C for 2½ hr.	854.1 ± 2.4*	846.4 ± 39.3*	29.9 ± 8.3*

usually determined) or further incubation for 2 hr in K^+ - free Krebs solution did not significantly affect the values further.

When Na^+ - rich tissues were transferred to a medium containing 4.6 mM K^+, the tissues rapidly extruded Na^+ and accumulated K^+. The time course of the recovery process of Na^+ - enriched tissues incubated in Krebs solution is illustrated in Figure 4. Tissues lost Na^+ and gained K^+ rapidly during the first 60 min, attaining a steady-state at about 2 hr. Subsequent experimental incubations were thus usually terminated at 2 hr. The total amount of Na^+ lost was about 250 mM/kg dry wt and was accompanied by an equivalent gain of K^+. The tissue water content remained unchanged throughout the incubation period.

The Effect on Recovery of Na^+ - Rich tissues of:-

A. Hormones

Figure 5 illustrates the recovery process of Na^+ - enriched tissues obtained from male rabbits, estrogen-dominant female rabbits, and progesterone-dominant female rabbits. Female rabbits had been injected for 4 days previous to sacrifice with either 100 µg diethylstilboestrol (estrogen-dominant), or 10 mg progesterone and 10 µg diethylstilboestrol (progesterone-dominant). The rate of Na^+ loss and K^+ gain was similar in all three cases, and the equilibration values for the tissues obtained from females at 2 hr and at 4 hr were not significantly different from the values for tissues obtained from males at those time periods.

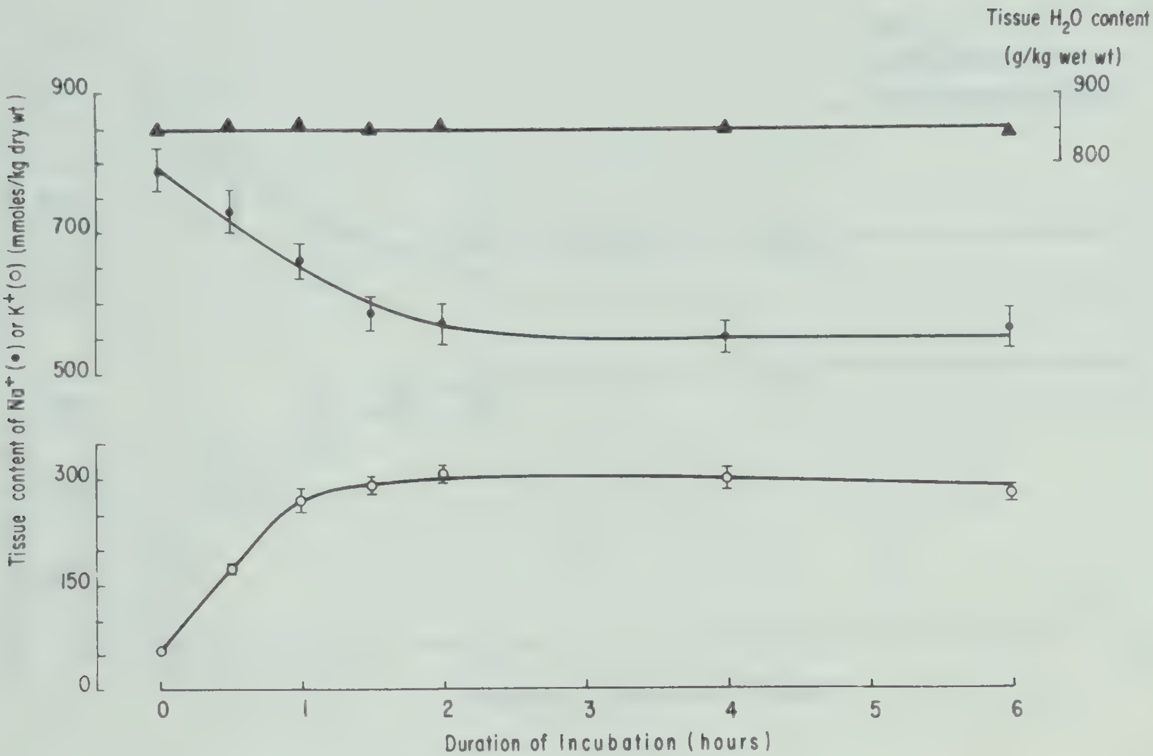

FIGURE 4. Recovery of Na^+ - rich rabbit detrusor muscle with time. Na^+ - rich tissues were pre-incubated for ½ hr. in K^+ - free Krebs solution at 37°C. At zero time, tissues were placed in Krebs solution, containing 4.6 mM K^+. The subsequent changes in the H_2O (▲), Na^+ (●) and K^+ (o) contents of the tissues are plotted against time. Each point is the mean ± S.E. of 15 - 36 observations.

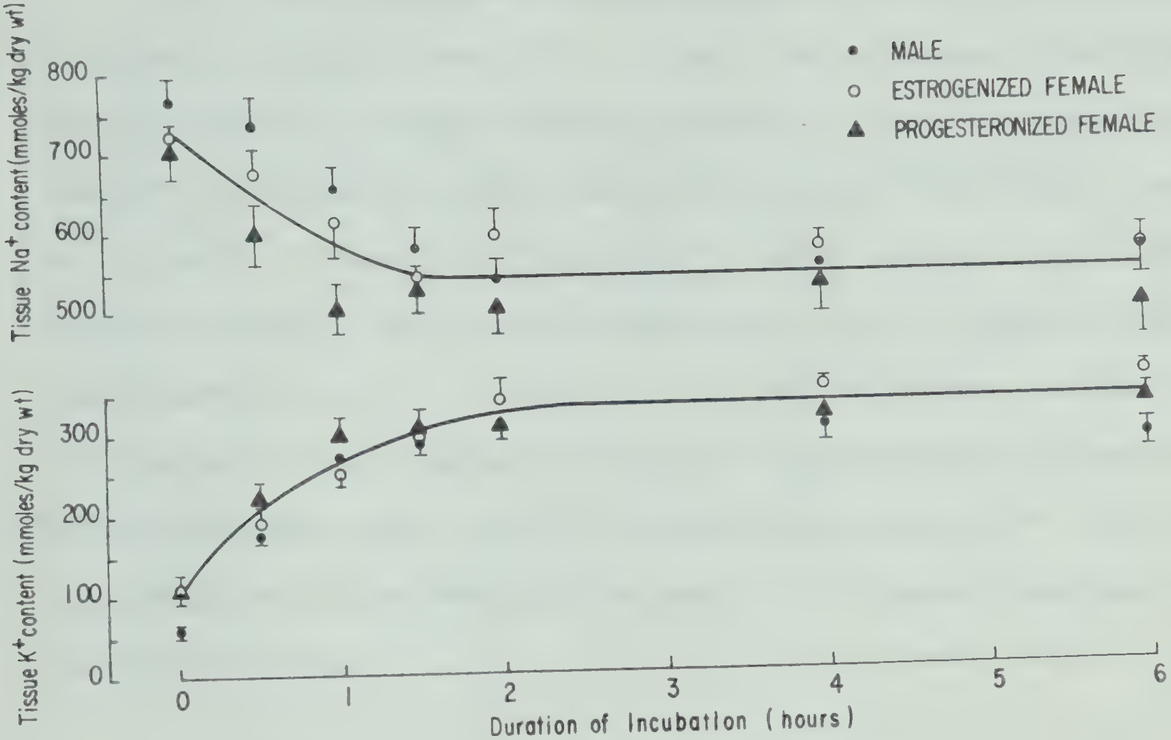

FIGURE 5. Effect of hormones on the recovery of Na^+ - rich tissues. Tissues were obtained from male rabbits (●), female rabbits under the influence of estrogen (o), or female rabbits under the influence of progesterone (▲). After Na^+ - enrichment, tissues were pre-incubated in K^+ - free Krebs solution for ½ hr. At zero time, tissues were placed in normal Krebs solution containing 4.6 mM K^+ and the subsequent changes in Na^+ and K^+ contents of the tissues plotted against time. Each point is the mean ± S.E. of 8 observations.

B. Temperature

Na^+-rich tissues were pre-incubated for ½ hr in K^+-free Krebs solution at 0°C, 27°C, and 37°C. At zero time in Figure 6, the tissues were transferred to Krebs solution containing 4.6 mM K^+, at the corresponding temperature, and the subsequent changes in the tissue contents of Na^+ and K^+ followed for 2 hr. At 0°C, the K^+ content of the tissues remained constant. Na^+ was lost during the first ½ hr but no further loss occurred up to 2 hr incubation. The rate of recovery of ions at 27°C was much slower than at 37°C, but at 2 hr there was no significant difference between the values at 27°C and the values at 37°C. The water content of the tissues remained the same in all tissues throughout the incubation period.

C. Ouabain

Tissues were pre-incubated for ½ hr in K^+-free Krebs solution containing varying concentrations of ouabain. 4.6 mM K^+ was then added to the medium and the incubation continued for another 2 hr. The difference between the Na^+ and K^+ contents of Na^+-rich tissues, and the ion contents of tissues incubated for 2 hr in Krebs solution in the absence of ouabain, was taken as 100%. The percentage of Na^+ lost and percentage of K^+ gained was then plotted against the log of the ouabain concentration (Figure 7). Inhibition of ion movements by ouabain was concentration-dependent, with Na^+ extrusion being inhibited to the same extent as K^+ accumulation. Maximal inhibition occurred at a concentration of 10^{-5} M. The ED_{50} for ouabain was about 4×10^{-7} M.

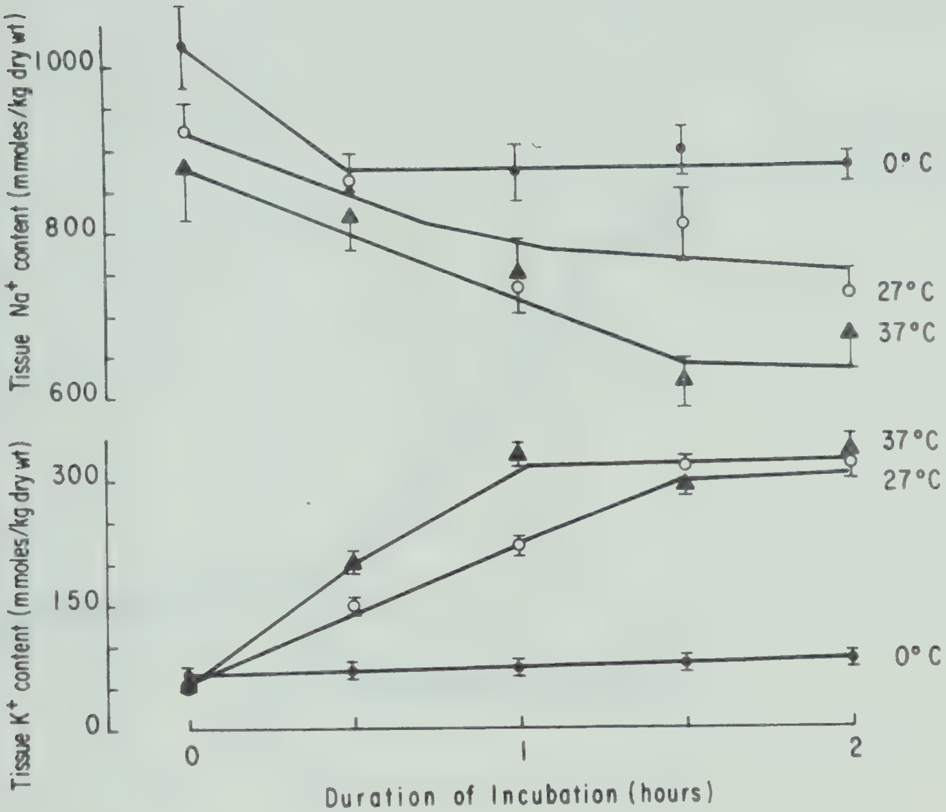

FIGURE 6. Effect of temperature on the recovery of Na^+ - rich rabbit detrusor muscle. Na^+ - rich tissues were pre-incubated for ½ hr. in K^+ - free Krebs solution at 0°C (●), 27°C (o) and 37°C (▲). At zero time 4.6 mM K^+ was added to the solutions. The subsequent changes in the Na^+ and K^+ contents of the tissues are plotted against time. Each point is the mean ± S.E. of 8 observations.

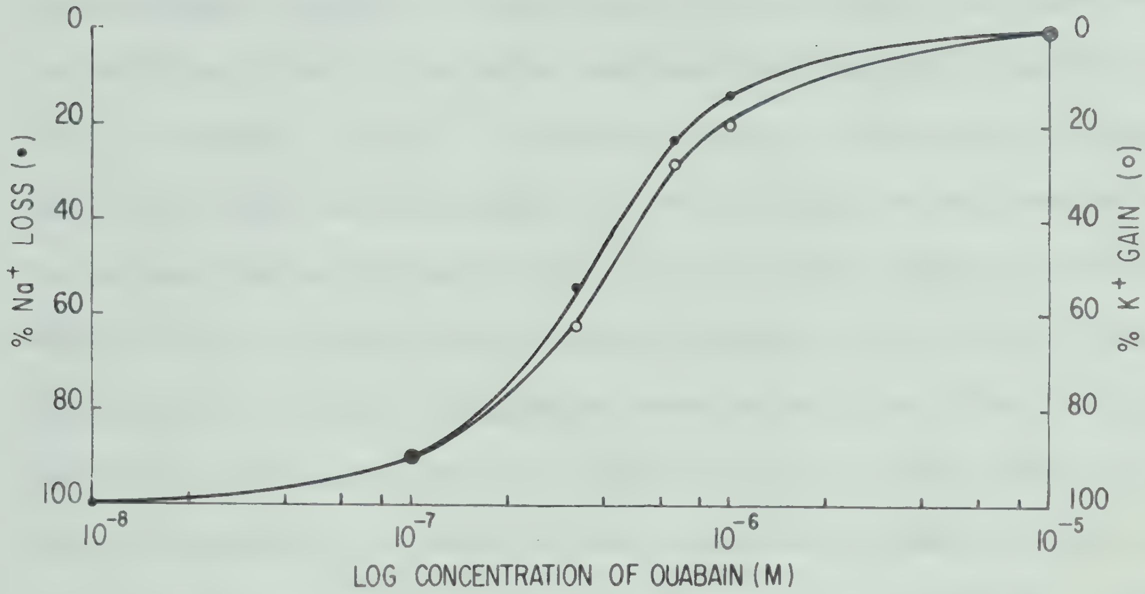

FIGURE 7. Effect of ouabain on the recovery of Na^+ - rich tissues. Na^+ tissues were pre-incubated for ½ hr. in K^+ - free solution containing varying concentrations of ouabain. The percentage of K^+ gained (o) and the percentage of Na^+ lost (•) is plotted against the log of the concentration of ouabain.

D. D-Glucose and Oxygen

The effect of D-glucose and of oxygen on the recovery of Na^+-enriched tissues was investigated by pre-incubating and incubating the tissues under conditions in which D-glucose was present or absent from the media and in which the media were equilibrated with either 95% O_2, 5% CO_2, or 95% N_2, 5% CO_2. As shown in Table 3, the absence of oxygen had little effect on the recovery of ions if D-glucose was present in the medium, indicating that glycolysis alone could support ion recovery. Ther was also no significant difference between the ion contents of tissues incubated with or without D-glucose under aerobic conditions. Thus oxidation of endogenous substrates was also able to supply sufficient energy to enable the recovery of ions to proceed. However, tissues incubated under anaerobic conditions, in the absence of D-glucose, showed marked impairment of ion recovery.

E. 2,4-Dinitrophenol (DNP)

Since the recovery of ions was shown to proceed unhindered under anaerobic conditions when glycolysis alone was operative, this phenomenon was investigated further with the use of the compound 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation as well as a stimulator of mitochondrial ATPase. The actions of this agent are reversible (Webb, 1966), and thus in the experiments performed with this agent, it was added to both the pre-incubation and the incubation media. When D-glucose was supplied as a glycolytic substrate, very little impairment of ion movements occurred with DNP (Table 4). DNP had a much greater inhibitory effect on K^+ accumulation than on Na^+ extrusion, the effect

TABLE 3

EFFECT OF OXYGEN AND D-GLUCOSE ON RECOVERY OF ION CONTENTS

Na^+ - rich tissues were pre-incubated for ½ hr. in K^+ - free Krebs solution with (+) or without (-) 10 mM D-glucose, the media being equilibrated with either 95% O_2 and 5% CO_2 (+) or 95% N_2 and 5% CO_2 (-), and were then incubated for 2 hr. in Krebs solution under the same conditions. Values are the mean ± S.E. for 24 - 30 observations. * indicates a value significantly different from the value for tissues exposed to D-glucose and to O_2.

TREATMENT		TISSUE CONTENT OF		
		H_2O	Na^+	K^+
D-GLUCOSE	OXYGEN	(g/kg wet wt)	(mmoles/kg dry wt)	(mmoles/kg dry wt)
+	+	853.1 ± 19.1	601.9 ± 20.4	290.5 ± 10.2
+	-	852.5 ± 2.7	615.9 ± 17.7	275.6 ± 8.1
-	+	849.3 ± 3.2	632.1 ± 17.3	267.4 ± 11.7
-	-	854.6 ± 3.7	811.0 ± 30.9*	94.0 ± 5.5*

EFFECT OF DINITROPHENOL ON RECOVERY OF ION CONTENTS

Na^+ - rich tissues were pre-incubated for ½ hr. in K^+ - free Krebs solution and then in Krebs solution for 2 hr. DNP and D-glucose were present (+) or absent (-) throughout, as indicated below. Values represent the mean ± S.E. of 4 - 14 observations. * indicates a value significantly different from the value in the absence of DNP. † indicates a value significantly different from the corresponding value in the presence of D-glucose.

TREATMENT		TISSUE CONTENT OF		
		H_2O	Na^+	K^+
DNP (mM)	D-glucose (10 mM)	(g/kg wet wt)	(mmoles/kg dry wt)	(mmoles/kg dry wt)
-	+	846.9 ± 4.3	594.4 ± 34.5	305.7 ± 9.8
0.1	+	855.0 ± 6.7	641.6 ± 50.5	228.7 ± 32.7
0.33	+	852.6 ± 3.4	688.4 ± 38.0	189.3 ± 8.6*
0.67	+	849.0 ± 3.8	649.0 ± 25.2	168.8 ± 8.6*
1.0	+	847.6 ± 3.9	669.8 ± 33.6	143.4 ± 5.3*
5.0	+	864.8 ± 4.7*	887.6 ± 14.0*	115.7 ± 7.3*
0.33	-	853.9 ± 7.2	890.3 ± 58.7*†	64.6 ± 4.0*†
1.0	-	846.8 ± 3.9	826.2 ± 18.0*†	51.1 ± 3.6*†

increasing as the concentration increased. A significant effect was seen on the extrusion of Na^+ at a concentration of 5 mM DNP, but at this concentration significant swelling also occurred. Both Na^+ extrusion and K^+ accumulation were completely inhibited in the presence of DNP when glucose was omitted from the media.

F. Azide

Azide is also an uncoupler of oxidative phosphorylation as well as an inhibitor of the cytochrome C system, but it does not have the ATPase stimulatory effect that DNP possesses (Webb, 1966). Tissues were pre-incubated and incubated in the presence of the indicated concentrations of azide (Table 5). Significant, but partial inhibition of the recovery process occurred only at 10 mM. The partial recovery which occurred in the presence of 10 mM azide was completely inhibited upon the removal of D-glucose from the media.

G. Glycolytic Substrates

The ability of various substrates to enter the glycolytic pathway and provide the energy requirements for ion pumping was studied under anaerobic conditions. Tissues were pre-incubated in K^+-free, glucose-free Krebs solution containing 20 mM of one of the following substrates: sucrose, D-glucose, D-mannose, D-galactose, D-xylose, D-fructose, D-arabinose, **L**-glucose, 3-o-methylglucose, 2-deoxy-D-glucose. 4.6 mM K^+ was then added to the medium and the tissues incubated for a further 2 hr. All media were equilibrated with 95% N_2, 5% CO_2. Results are shown in Table 6. Of the various monosaccharides used, only D-glucose and D-mannose were able to supply sufficient energy to enable Na^+

TABLE 5

EFFECT OF AZIDE ON RECOVERY OF ION CONTENTS

Na^+ - rich tissues were pre-incubated for ½ hr. in K^+ - free Krebs solution and then in Krebs solution for 2 hr. Azide and D-glucose were present (+) or absent (-) throughout, as indicated above. Values represent the mean ± S.E. of 12 observations. * indicates a value significantly different from the value in the absence of azide. † indicates a value significantly different from the corresponding value in the presence of D-glucose.

TREATMENT		TISSUE CONTENT OF		
Azide (mM)	D-glucose (10 mM)	H_2O (g/kg wet wt)	Na^+ (mmoles/kg dry wt)	K^+ (mmoles/kg dry wt)
-	+	860.1 ± 9.1	720.5 ± 57.6	353.6 ± 22.3
1.0	+	868.4 ± 5.2	726.4 ± 34.4	315.6 ± 16.0
10.0	+	870.0 ± 4.0	830.9 ± 38.1*	256.9 ± 11.8*
10.0	-	846.6 ± 7.5†	891.6 ± 60.5*	53.8 ± 4.8*†

TABLE 6

EFFECT OF GLYCOLYTIC SUBSTRATES ON RECOVERY OF ION CONTENTS UNDER ANAEROBIC CONDITIONS

Na^+ - rich tissues were pre-incubated for ½ hr. in K^+ - free Krebs solution containing the substrate indicated and 4.6 mM K^+ then added for an additional 2 hr. All media were equilibrated with 95% N_2 and 5% CO_2. Values represent the mean ± S.E. of 8 - 18 observations. * indicates a value significantly different from the value obtained using sucrose.

SUBSTRATE (20 mM)	TISSUE CONTENT OF H_2O (g/kg wet wt)	Na^+ (mmoles/kg dry wt)	K^+ (mmoles/kg dry wt)
Sucrose	861.9 ± 4.2	953.9 ± 35.6	51.2 ± 3.5
D-glucose	859.3 ± 4.4	666.2 ± 34.2*	282.0 ± 16.2*
D-mannose	871.5 ± 4.5	787.4 ± 44.8*	267.4 ± 15.5*
D-galactose	874.6 ± 4.1	1066.7 ± 51.6	53.9 ± 1.4
D-xylose	877.1 ± 3.3*	1101.0 ± 46.7*	53.5 ± 3.4
D-fructose	872.1 ± 2.6	1028.0 ± 50.1	62.5 ± 3.6
D-arabinose	869.7 ± 8.7	1078.7 ± 84.6	45.1 ± 6.6
L-glucose	856.2 ± 6.9	999.9 ± 65.8	49.0 ± 3.5
3-o-methyl-glucose	850.7 ± 5.8	893.0 ± 30.5	42.8 ± 4.2
2-deoxy-D-glucose	861.8 ± 9.8	968.7 ± 84.1	50.7 ± 7.6

extrusion and K^+ accumulation to occur. Some swelling, accompanied by a further increase in the tissue Na^+ content, occurred with D-galactose, D-xylose, D-fructose, and D-arabinose, although this was significant only in the case of D-xylose.

H. 2-Deoxy-D-Glucose (2DG)

In order to further investigate the importance of glycolysis in the maintenance of the ionic composition of the rabbit detrusor muscle, 2-deoxy-D-glucose was used as a potential glycolytic inhibitor, as it has been demonstrated in several tissues to block glycolysis (Webb, 1966). 20 mM of either sucrose, D-glucose or 2DG were used as substrates throughout the pre-incubation and incubation periods and the media were equilibrated with 95% O_2, 5% CO_2. Tissues incubated in the presence of 2DG were unable to extrude Na^+ and accumulate K^+ to the same extent as tissues incubated in the presence of D-glucose (Table 7). This inhibitory effect of 2DG was not, however, significantly different from the effect on ion contents of tissues incubated in the presence of sucrose (glucose-free media). Thus under oxidative conditions 2DG did not inhibit the supply of energy for ion movements to any greater extent than that caused by the omission of a suitable substrate. A similar experiment was performed under anaerobic conditions (Table 8). The presence of 2DG alone caused no greater inhibitory effect on ion recovery than the absence of D-glucose (sucrose-substituted). However, when 2DG and D-glucose were added simultaneously to the media, an inhibitory effect on ion movements, significantly greater than the inhibition caused by the combination of sucrose and D-glucose added simultaneously to the media, was noted. That is,

TABLE 7

EFFECT OF 2-DEOXY-D-GLUCOSE ON THE RECOVERY OF ION CONTENTS UNDER AEROBIC CONDITIONS

Tissues were pre-incubated for ½ hr. in K^+ -free Krebs solution, then in Krebs solution for 2 hr. The substrates (20 mM) indicated were present throughout. All media were equilibrated with 95% O_2 and 5% CO_2. Values represent the mean ± S.E. of 20 observations. * indicates a value significantly different from that obtained with 20 mM D-glucose.

SUBSTRATE (20 mM)	TISSUE CONTENT OF		
	H_2O (g/kg wet wt)	Na^+ (mmoles/kg dry wt)	K^+ (mmoles/kg dry wt)
Sucrose	851.3 ± 2.8	655.1 ± 23.7	292.3 ± 11.3
D-glucose	859.2 ± 3.7	607.0 ± 24.6	307.9 ± 12.9
2-deoxy-D-glucose	860.9 ± 2.4	737.1 ± 35.4*	254.0 ± 15.1*

TABLE 8

EFFECT OF 2-DEOXY-D-GLUCOSE (2DG) ON THE RECOVERY OF ION CONTENTS UNDER ANAEROBIC CONDITIONS

Tissues were handled as described in Table 7 except that all media were equilibrated with 95% N_2 and 5% CO_2. Values represent the mean ± S.E. of 8 - 20 observations. * indicates a value significantly different from that obtained with 20 mM D-glucose. † indicates a value significantly different from the corresponding value in the absence of 2DG.

SUBSTRATE	TISSUE CONTENT OF H_2O (g/kg wet wt)	Na^+ (mmoles/kg dry wt)	K^+ (mmoles/kg dry wt)
20 mM D-glucose	856.1 ± 2.8	619.5 ± 21.4	298.4 ± 5.7
20 mM sucrose	862.0 ± 3.2	912.0 ± 27.3*	86.5 ± 8.0*
20 mM 2DG	863.8 ± 7.8	941.2 ± 51.2*	76.7 ± 5.3*
5 mM D-glucose + 15 mM sucrose	849.9 ± 6.0	712.0 ± 46.3*	223.3 ± 13.3*
5 mM D-glucose + 15 mM 2DG	861.6 ± 5.0	922.9 ± 38.1*†	99.1 ± 7.5*†
10 mM D-glucose + 10 mM sucrose	854.0 ± 4.2	680.2 ± 36.8	258.0 ± 12.1
10 mM D-glucose + 10 mM 2DG	869.0 ± 2.6*†	918.5 ± 26.2*†	107.0 ± 2.6*†

whereas 5 mM D-glucose (and 15 mM sucrose) were able to produce a substantial extrusion of Na^+ and accumulation of K^+ under anoxic conditions, 10 mM of 2DG was able to effectively compete with as high a concentration of D-glucose as 10 mM and thus completely prevent the recovery of ions.

I. N-Ethyl Maleimide (NEM)

N-ethyl maleimide is an agent which has a high selectivity for -SH groups and thus may react with a variety of enzymes. It was used as a possible inhibitor of glycolytic enzymes by limiting the duration of exposure of the tissues to this agent. Thus tissues were pre-incubated in K^+-free Krebs solution containing the indicated concentrations of NEM (Table 9) for 30 min only. Tissues were then transferred to Krebs solution containing 4.6 mM K^+, but no inhibitor, for a further 2 hr incubation period. Inhibition produced by NEM was concentration-dependent; a fairly high concentration (0.67 mM) was required to produce a significant inhibitory effect on ion movements. A significant amount of swelling occurred at a concentration of 1 mM NEM. The substitution of 20 mM pyruvate for D-glucose in the medium proved unable to reverse the effects of NEM, indicating that this agent was probably not selectively inhibiting glycolysis.

J. Iodoacetic Acid (IAA)

The effects on ion recovery of another sulfydryl reagent, iodoacetic acid (IAA), were examined. This agent, if used in small concentrations, is a relatively selective inhibitor of the enzyme phosphogly-

TABLE 9

EFFECTS OF N-ETHYLMALEMIDE (NEM) ON THE RECOVERY OF ION CONTENTS

Na^+ - rich tissues were incubated for ½ hr. in K^+ - free Krebs solution containing the indicated concentration of NEM, then for 2 hr. in Krebs solution alone. All solutions contained 10 mM d-glucose unless otherwise indicated. Values represent the mean ± S.E. of 10 - 20 tissues. * indicates a value significantly different from the value obtained in the absence of the inhibitor.

NEM (mM)	TISSUE CONTENT OF H_2O (g/kg wet wt)	Na^+ (mmoles/kg dry wt)	K^+ (mmoles/kg dry wt)
-	850.2 ± 5.6	658.0 ± 42.1	251.8 ± 17.5
0.33	858.1 ± 9.7	813.0 ± 81.6	215.1 ± 26.6
0.67	866.7 ± 10.3	906.5 ± 67.7*	168.2 ± 12.0*
1.0	869.2 ± 6.1*	965.8 ± 77.1*	102.6 ± 8.4*
1.0 (+ 20 mM pyruvate)	856.7 ± 4.7	928.7 ±141.7*	78.0 ± 12.7*

ceraldehyde dehydrogenase, but inhibits a variety of sulfur-containin enzymes if used in higher concentrations and/or for long periods of time (Webb, 1966). For this reason, tissues were pre-incubated in K^+-free Krebs solution containing the indicated concentrations of IAA (see Table 10) for 30 min only, and then in Krebs solution without the inhibitor for a subsequent 2 hr. Control tissues were not exposed to IAA. The inhibition of ion recovery by IAA was concentration-dependent, as little as 0.33 mM causing a significant effect. IAA also increased the water content of the tissues significantly. This swelling was accompanied by an apparently greater inhibitory effect on Na^+ extrusion than on K^+ accumulation. 1 mM IAA reduced Na^+ extrusion by 350 mM/kg dry wt but only reduced K^+ accumulation by 90 mM/kg dry wt. The partial impairment of ion recovery produced by 0.33 mM IAA was examined further by incubating poisoned tissues in the presence or absence of D-glucose under aerobic or anaerobic conditions (Table 11). The absence of D-glucose had no further inhibitory effects on ion recovery, suggesting that the lack of total inhibition produced by 0.33 mM IAA was not due to an incomplete inhibition of the enzyme phosphoglyceraldehyde dehydrogenase. Incubation under anaerobic conditions, after exposure to 0.33 mM IAA, had a significantly greater inhibitory effect on ion movements than exposure to 0.33 mM IAA under aerobic conditions. This caused marked tissue swelling accompanied by a further increase in the tissue Na^+ content. That is, 289 mM/kg dry wt of Na^+ were gained over and above that gained due to 0.33 mM IAA under aerobic conditions, with a concomitant reduction in K^+ accumulation of only 124 mM/kg dry wt.

TABLE 10

EFFECT OF IODOACETIC ACID (IAA) ON THE RECOVERY OF ION CONTENTS

Na^+ - rich tissues were incubated for ½ hr. in K^+ - free Krebs solution in the presence or absence of IAA, then in inhibitor-free Krebs solution for 2 hr. All solutions contained 10 mM d-glucose unless otherwise indicated. Values represent the mean ± S.E. of 8 - 20 observations.
* indicates a value significantly different from the value obtained in the absence of IAA.
† indicates a value significantly different from the value in 1 mM IAA + glucose.

IAA (mM)	TISSUE CONTENT OF					
	H_2O (g/kg wet wt)		Na^+ (mmoles/kg dry wt)		K^+ (mmoles/kg dry wt)	
-	850.1	4.0	608.0	25.1	290.9	12.1
0.33	863.3	3.9*	781.8	25.4*	213.3	13.5*
0.67	868.0	12.8*	913.5	10.6*	169.3	25.7*
1.00	863.1	4.2*	958.3	44.6*	99.1	6.3*
5.00	878.9	4.5*	1038.0	59.1*	75.8	4.4*
1.00 (+ 20 mM pyruvate)	842.2	5.6	681.4	27.2†	189.5	14.5*†

TABLE 11

FACTORS INFLUENCING THE EFFECT OF IODOACETIC ACID ON THE RECOVERY OF ION CONTENTS

Na^+ - rich tissues were incubated for ½ hr. in the presence (+) or absence (-) of 0.33 mM IAA, then in inhibitor-free Krebs solution for 2 hr. Media contained 10 mM D-glucose (+) or sucrose (-) and were equilibrated with 95% O_2 and 5% CO_2 (+) or 95% N_2 and 5% CO_2 (-). Values represent the mean ± S.E. of 12 - 16 observations. * indicates a value significantly different from the value in the absence of IAA. † indicates a value significantly different from the value in the presence of IAA, D-glucose and O_2.

TREATMENT			TISSUE CONTENT OF		
			H_2O	Na^+	K^+
0.33 mM IAA	10 mM D-glucose	O_2	(g/kg wet wt)	(mmoles/kg dry wt)	(mmoles/kg dry wt)
-	+	+	849.9 ± 6.0	641.3 ± 30.4	283.3 ± 14.0
+	+	+	870.0 ± 5.1*	920.2 ± 57.2*	173.2 ± 18.4*
+	-	+	865.3 ± 4.6	915.7 ± 48.6*	177.9 ± 13.6*
+	+	-	884.2 ± 7.1*	1209.1 ± 68.7*†	49.0 ± 3.2*†

K. Oxidative Substrates

Various substrates were tested as to their ability to enter the Krebs cycle and provide sufficient energy for the recovery of the ion contents of the Na^+-rich rabbit detrusor muscle when glycolysis was inhibited with 1 mM IAA. Tissues were pre-incubated for ½ hr in K^+-free, glucose-free Krebs solution containing 1 mM IAA and 20 mM of one of the following substrates: D-glucose, pyruvate, lactate, oxaloacetate, β-hydroxybutyrate, succinate, L-alanine, α-ketoglutarate, α-ketoglutarate plus L-alanine (10 mM each). β-hydroxybutyrate was used as the mono-sodium salt and succinate as the disodium salt, an equivalent amount of NaCl was being omitted from the medium. Oxaloacetate was used as the acid and the necessary pH adjustments made. Tissues were then transferred to glucose-free Krebs solution containing one of the substrates and incubated for a further 2 hr. A control set of tissues was incubated in 20 mM D-glucose in the absence of IAA. As can be seen in Table 12, pyruvate, lactate, oxaloacetate and β-hydroxybutyrate were all capable of partially supporting ion pumping after exposure to IAA. These substrates were also able to reverse, to some extent, the swelling which occurred with IAA. It has been demonstrated in rat liver slices (Siedman and Cascarano, 1966) that α-ketoglutarate plus L-alanine could provide energy for ion pumping, presumably due to their conversion to pyruvate. This combination, however, had no such effect in the rabbit detrusor muscle.

TABLE 12

EFFECT OF VARIOUS SUBSTRATES ON RECOVERY OF
ION CONTENTS AFTER TREATMENT WITH 1 mM IODOACETIC ACID

Na^+ - rich tissues were incubated for ½ hr. in K^+ - free, glucose-free Krebs solution with the designated substrate and 1 mM IAA, then in glucose-free Krebs solution plus substrate for 2 hr. Values represent the mean ± S.E. of 8 - 23 tissues. * indicates a value significantly different from the value obtained with glucose after IAA.

SUBSTRATE (20 mM)	TISSUE CONTENT OF H_2O (g/kg wet wt)	Na^+ (mmoles/kg dry wt)	K^+ (mmoles/kg dry wt)
Glucose (no IAA)	845.9 ± 4.8	533.5 ± 20.5*	303.7 ± 13.3*
D-glucose	863.9 ± 5.2	901.7 ± 48.2	116.2 ± 12.3
Pyruvate	862.8 ± 4.7	759.9 ± 47.2*	220.3 ± 19.2*
Lactate	845.4 ± 8.2	650.8 ± 35.2*	216.0 ± 25.9*
Oxaloacetate	854.5 ± 1.2	629.7 ± 41.2*	225.9 ± 21.5*
β-Hydroxybutyrate	851.9 ± 4.4	574.8 ± 20.7*	219.7 ± 28.7*
Succinate	863.8 ± 2.5	776.0 ± 28.0	141.8 ± 19.1
ℓ-Alanine	870.8 ± 3.8	937.8 ± 39.3	136.1 ± 17.6
α-Ketoglutarate	863.3 ± 4.1	847.4 ± 35.3	137.0 ± 14.3
α-Ketoglutarate + ℓ-Alanine	858.6 ± 4.6	884.2 ± 39.7	133.3 ± 14.3

L. Oxidative Substrates Under Anaerobic Conditions

Siedman and Cascarano (1966) demonstrated that addition of oxaloacetate or lactate to the incubation medium allowed coupled Na^+-K^+ pumping to occur in anoxic rat liver slices. These workers suggested that these substrates accepted electrons and facilitated the subsequent production of NADP, which in turn stimulated ATP production via glycolysis as well as mitochondrial ATP production. A number of substrates were tested in the detrusor muscle to see if this phenomenon would occur. Tissues were pre-incubated in K^+-free glucose-free Krebs containing 20 mM of one of the following substrates: D-glucose, pyruvate, β-hydroxybutyrate, α-ketoglutarate, succinate, lactate, oxaloacetate; followed by 2 hr incubation in K^+-containing Krebs solution plus the substrate. All media were equilibrated with 95% N_2, 5% CO_2. Table 12 shows that none of the oxidative substrates used enabled the tissues to carry on ion pumping under anaerobic conditions.

TABLE 13

EFFECT OF OXIDATIVE SUBSTRATES ON RECOVERY OF ION CONTENTS UNDER ANAEROBIC CONDITIONS

Na^+ - rich tissues were incubated for ½ hr. in K^+ - free glucose-free Krebs solution, then for 2 hr. in glucose-free Krebs. The indicated substrate was present throughout. All media were equilibrated with 95% N_2 and 5% CO_2 throughout. Values represent the mean ± S.E. of 8 tissues. * indicates a value significantly different from the value obtained with D-glucose.

SUBSTRATE (20 mM)	TISSUE CONTENT OF H_2O (g/kg wet wt)	Na^+ (mmoles/kg dry wt)	K^+ (mmoles/kg dry wt)
D-glucose	859.5 ± 8.7	618.0 ± 43.2	246.8 ± 24.1
Pyruvate	853.7 ± 7.2	793.7 ± 38.8*	66.1 ± 10.9*
β-Hydroxybutyrate	871.9 ± 5.4	930.6 ± 42.0*	66.6 ± 9.0*
α-Ketoglutarate	850.3 ± 7.7	720.3 ± 34.8	78.9 ± 12.5*
Succinate	874.6 ± 5.3	953.7 ± 35.5*	61.5 ± 9.4*
Lactate	878.5 ± 9.1	1039.1 ± 78.3*	68.1 ± 13.9*
Oxaloacetate	873.0 ± 9.4	888.8 ± 57.7*	81.8 ± 14.7*

IV. DISCUSSION

DISCUSSION

A. Extracellular Space

The measurement of the volume of the extracellular space of a tissue with the use of a radioactive tracer molecule is based on several assumptions:

(1) the concentration of the tracer molecule in the extracellular space is equal to the concentration of the molecule in the incubating medium,

(2) the tracer molecule occupies the whole of the extracellular space,

(3) the tracer molecule does not penetrate the cells,

(4) the tracer molecule is not fixed by any extracellular constituent (see Schoffeniels, 1967).

If these criteria hold true, different impermeant molecules should all be able to equilibrate to the same volume of distribution. However, as shown by the variability in values for the extracellular space as reported by Burnstock et al. (1963), Goodford (1968), and Burnstock (1970), few of the tracer molecules used possess all these characteristics.

There is considerable evidence that much of the extracellular fluid of smooth muscle is subdivided by a meshwork of collagen (Goodford, 1968) and/or mucopolysaccharides (Caesar et al., 1957; Villamil et al., 1968) which may exclude a molecule, by virtue of its size or charge, from areas of the extracellular space. Alternatively, some molecules may bind to these or other extracellular components. Molecules in the former category would underestimate the size of the extracellular space, whereas molecules which bind would tend to overestimate the extracellular space. Ogston and Phelps (1961) suggested that large molecules such as inulin

may be excluded from parts of the extracellular space because of the steric hindrance caused by mucopolysaccharides such as hyaluronic acid. The treatment of guinea pig taenia coli with hyaluronidase increased the inulin space (Goodford and Leach, 1964) in accordance with this theory, without significantly affecting the ionic contents and wet weight of the tissue. Osman (1971) observed a shrinkage of the rabbit detrusor muscle upon incubation with hyaluronidase, and a decrease in the volume of distribution of the extracellular markers used. Osman suggested that any increase in the volume of the spaces due to the degradation of hyaluronic acid by hyaluronidase may have been negated by the shrinkage of the tissue.

The problem of intracellular permeation of extracellular markers has been well documented. Bozler and Lavine (1958) presented evidence to suggest that sucrose penetrated the cell membrane of frog stomach muscle, and in 1961 Bozler suggested that many of the non-metabolizable sugars, used for the purpose of extracellular marking, were able to penetrate muscle. Daniel and Robinson (1971,b) have also shown that sucrose appears to be able to enter the cells of the rat uterus. Efflux of sucrose, dextran and inulin from frog stomach muscle (Bozler, 1966) and efflux of inulin and mannitol from rabbit detrusor muscle (Osman, Munson and Paton, 1971) was shown to be multicompartmental in nature, with the slowly effluxing compartment believed to be intracellular.

Further problems arise in the estimation of the volume of the extracellular space because of the possibility that tissues damaged during dissection procedures will give an overestimate of the value for the extracellular space. Stephenson (1971) has demonstrated in frog muscle that molecules such as sucrose and inulin are able to equilibrate with the

water of damaged cells at the tissue margins, yielding significantly higher values for the extracellular space than tissues not damaged by dissection before equilibration.

The present study emphasizes the problems involved in estimating the volume of the extracellular space in smooth muscle as reviewed by Goodford (1968). Some evidence was obtained that there appeared to be an inverse correlation between the molecular weight of the tracer molecule used and its volume of distribution in the tissue, smaller molecular weight compounds (e.g., sucrose and mannitol) equilibrating to a larger distribution ratio than the higher molecular weight compounds (inulin and dextran). Similar findings have been reported for guinea-pig taenia coli (Goodford and Leach, 1964), canine intestinal circular muscle (Barr and Malvin, 1965) and canine carotid arterial muscle (Villamil et al., 1968), whereas Weiss (1966) reported that sucrose, inulin and mannitol all gave similar values for the extracellular space of the longitudinal muscle of the guinea-pig ileum.

Two distinctly different equilibration values were obtained in the rabbit detrusor muscle; that reached by sucrose and mannitol was about 60 ml/100 g, and that reached by inulin, light and heavy dextran was about 45 ml/100 g. The equilibration of mannitol and sucrose to about 70% of the total tissue water suggests that these molecules may have penetrated intracellularly. This suggestion is further emphasized under conditions in which metabolism was inhibited, whereby the volume of distribution of mannitol approached that of the total tissue water, that is almost 85 ml/100 g. The volume of distribution of inulin was also increased in glucose-free medium, in the presence of IAA and DNP. It is probable that the severe

metabolic inhibition produced by these agents may have destroyed the semi-permeable character of the plasma membrane and thus allowed both inulin and mannitol to penetrate to a greater extent. Osman (1971) also showed that metabolic inhibition increased the spaces occupied by mannitol and sucrose in rabbit detrusor muscle, while the spaces occupied by inulin and dextran were not significantly altered. Metabolic inhibition produced by IAA and DNP has been shown to cause an increase in the weight of the tissue (Daniel and Robinson, 1971; Osman and Paton, 1971) and it may be argued that the changes in the spaces occupied by the latter compounds were masked by this concomitant gain in tissue weight. Daniel and Robinson (1971,a) also found that in rat uterus the swelling caused by metabolic inhibition of fresh tissues was reflected by an increase in the size of the ^{14}C-sucrose space but a decrease in the size of the space occupied by ^{3}H-inulin. They attributed this to an increased permeability of the cell membrane to sucrose but not to the larger inulin molecule. Villamil and his Coworkers (1968) however, found an increase in the inulin space on exposure of the canine carotid artery to sodium iodoacetate plus sodium cyanide, as well as total equilibration of sucrose with the tissue water under these conditions.

Ouabain had no significant effect on the size of the extracellular space as measured by inulin or mannitol. Although this agent caused a gain in the Na^+ content of the tissue and a loss of K^+, it did not affect the total water content of the tissue (Osman and Paton, 1971); that is, swelling or shrinkage of the tissue did not occur. Similar results were reported by Casteels (1966) for guinea-pig taenia coli, but under these conditions Casteels found an increase in the size of the ethanesulfonate space. Daniel

and Robinson (1971,a) reported that ouabain-treated rat uterine tissues lost weight but that no significant differences in the ^{14}C-sucrose space was noted.

The results of the calculations of the intracellular concentrations of Na^+ and K^+ are reasonably good indices of the markers which best evaluate the size of the extracellular space. The lower molecular weight compounds such as mannitol and sucrose give values for the extracellular space which are nearly the same (mannitol) or larger (sucrose) than the extracellular Na^+ space, resulting in a very low, or negative intracellular Na^+ concentration. Any such estimate of the size of the extracellular space must be rejected. Barr and Malvin (1965) demonstrated a similar effect with arabinose and mannitol in the canine intestine.

Headings _et al_. (1960) provided evidence that Na^+ was bound in the extracellular space of the canine carotid artery. If Na^+ is bound to negatively charged groups in the paracellular matrix, as evidence recently presented by Palaty _et al_. (1969) and Jones and Karreman (1969) suggested, the amount of Na^+ in the extracellular space would be underestimated, resulting in an overestimate of the intracellular concentration of Na^+. In this case the values as given by the smaller molecular weight compounds for the volume of the extracellular space are even more in error.

It would appear that no ideal marker exists for accurately determining the size of the extracellular space, thus the choice of an extracellular marker remains up to the investigator concerned. Barr and Malvin (1965) felt that sucrose, raffinose and inulin, which all equilibrated to the same extent in the dog jejunum, were adequate estimators of the size of the extracellular space, whereas mannose and arabinose tended

to diffuse intracellularly as the incubation time increased. Villamil et al. (1968), however, felt that inulin underestimated the ECS in the canine carotid artery, whereas sucrose was the best marker because its distribution volume approached that of the total tissue water after metabolic inhibition. Schoffeniels (1967) chose inulin as his extracellular marker because he felt that he had shown that this molecule satisfied the criteria set up at the beginning of this section. According to the results of the present study it would appear that inulin and light dextran may give the most reasonable estimates of the size of the extracellular space of the rabbit detrusor muscle. Both of these compounds, however, are not homogeneous; the same compound supplied from different commercial sources may give different values for the extracellular space in the same tissue (Phelps, 1965; Levi, 1969). Care must therefore be exercised in the use of these compounds.

B. The Sodium Pump

The present study has characterized a coupled Na^+-K^+ pump in the rabbit detrusor muscle which possesses characteristics common to the sodium pumps in many other tissues:

(a) potassium is required in the external medium to "turn on" this sodium pump;

(b) this sodium pump is temperature sensitive;

(c) ouabian inhibits this sodium pump; and

(d) this sodium pump is dependent on metabolism.

Evidence has also been presented in this study to suggest that a mechanism other than the coupled Na^+-K^+ pump may be operating to control the intra-

cellular Na^+ and water contents of this tissue. Each of these points will now be discussed.

During the study of net ion movements in the Na^+-rich rabbit detrusor muscle, it was noted that alterations in the K^+ contents of the tissue were a better indicator of the operation of a coupled Na^+-K^+ pump than alterations in the Na^+ content, as the latter was often masked by tissue swelling or shrinkage. In this study, the ion contents of tissues have been expressed in terms of the dry weight of the tissue since the wet weight of smooth muscles is very susceptible to changes in the water content of tissues. As this study has shown, metabolic inhibitors caused a gain of Na^+ and water in addition to inhibiting coupled Na^+-K^+ pumping. Similar findings have been reported for the rat uterus (Rangachari et al., 1971; Daniel and Robinson, 1971,b). Other possible methods of expression would be in terms of either the fresh weight of the muscle measured immediately after the animal is killed, or the nucleic acid content of each sample (Goodford, 1970) as such reference parameters are not influenced by the experimental conditions to which the muscle is subsequently subjected.

There is some evidence that diethylstilboestrol may affect the activity of the (Na^++K^+)-stimulated ATPase (Karmaker, 1969). Smith and Stultz (1971) have also shown that diethylstilboestrol affects the transport of 2DG. Although no evidence was found in this study to suggest that estrogen or progesterone significantly affected the activity of the Na^+-K^+ pump, possible effects of these hormones on the actions of the drugs used in this study were eliminated with the use of male rabbits.

The evidence for the existence of a sodium pump similar to that

found in many other tissues was as follows:

1. Extrusion of Na^+ from Na^+-rich tissues required the presence of K^+ in the external medium. Like many other smooth muscles, (see Daniel, 1958; Goodford, 1962; Kao and Siegman, 1963; Hagemeijer *et al.*, 1965) the rabbit detrusor muscle has a higher Na^+ content and lower K^+ content than striated muscle. Upon incubation in a K^+-free medium, when metabolism was inhibited by a low temperature (4°C), ions moved down their concentration gradients (Na^+ gain; K^+ loss) until the tissue ion contents and the medium were in equilibrium. During this period of Na^+-enrichment, more Na^+ was gained by the tissue than K^+ was lost. This gain in Na^+ was accompanied by an increase in the total water content of the tissue. However, upon rewarming of the Na^+-rich tissues and subsequent incubation in a K^+-containing Krebs solution at 37°C, Na^+ was extruded from the tissue and an equimolar amount of K^+ was gained, while no significant changes occurred in the tissue water content. That is, the water gained during Na^+-enrichment was not extruded during the recovery of the ion contents. The ability of K^+ to turn on the Na^+ pump has been reported for other smooth muscles (Daniel and Robinson, 1960,a; Friedman *et al.*, 1968). Daniel and Robinson (1971,a) noted that rat uterine tissues which had swollen during Na^+-enrichment rapidly lost Na^+ and water when rewarmed in K^+-free medium at 25°C. Daniel and Robinson suggested that K^+-free solutions, while inhibiting the activity of the Na^+ pump, did not affect mechanisms involved in net sodium and water movements out of swollen Na^+-rich tissues.

2. Ion movements in Na^+-rich tissues were sensitive to temperature. Ion recovery was inhibited at 0°C, while significant ion movements occurred at 27°C as well as at 37°C. Although the process of ion recovery was not

significantly different between 27°C and 37°C, this was not an unusual phenomenon in smooth muscle. Daniel and Robinson (1960,b) also reported active movements of Na^+ and K^+ in cat and rabbit uterine segments at 24°C. Similar results were reported for rabbit uterus by Kao and Siegman (1963). This does not eliminate the possibility that these movements were active, for it is possible that a reduction in temperature results in a slowing of the passive movements of Na^+ and K^+ down their electrochemical gradients, which compensated for any decrease of active transport. More recently, Daniel and Robinson (1970) have reported that both efflux and influx of Na^+ from the rat uterus were dependent on temperature, to an extent expected of a diffusion controlled process. The rate of cellular Na^+ efflux did not decrease to any great extent from 37°C to 15°C but rapidly decreased between 15°C and 5°C. Daniel and Robinson thus concluded that although this low Q_{10} does not exclude an ion exchange process as the rate determining step, it would appear that the step which is rate-limiting does not involve the combination of Na^+ with a carrier or enzymatic phosphorylation, for this would involve a chemical reaction of high energy of activation. The authors suggested that a conformational change of membrane proteins may occur at 15°C which would account for the sudden slowing of efflux.

3. <u>Coupled Na^+-K^+ movements were inhibited by the cardiac glycoside ouabain.</u> In the Na^+-rich detrusor muscle the concentration of ouabain necessary to effectively inhibit any subsequent extrusion of Na^+ and accumulation of K^+, when incubated in a normal Krebs medium, was 10^{-5}M and the concentration required for 50% inhibition was 4×10^{-7}M. Similar concentrations of ouabain were required in other tissues to inhibit Na^+ and K^+ fluxes (see Glynn, 1964, for review). In the present set of

experiments, ouabain was present in the K^+-free pre-incubation media. There is some evidence that K^+ can overcome or prevent the inhibitory effect of ouabain (Matsui and Schwarz, 1968). Glynn (1956), and Matsui and and Schwarz (1966) have shown that this effect of K^+ cannot be explained on the basis of simple competitive kinetics. Recently Allen and Schwarz (1970) have suggested that K^+ acts at a site allosteric to the ouabain site. By completing the turnover cycle of the (Na^+-K^+)-stimulated ATPase, potassium would thus prevent the binding of ouabain and its subsequent inhibition of the enzyme. It is possible that if the drug was added only to the media containing K^+, a higher concentration of ouabain would be required for a given degree of inhibition.

4. <u>The Na^+-pump depends on metabolism for its supply of energy</u>.

(a) The role of glycolysis

The omission of a suitable substrate from the media completely inhibited ion movements when tissues were incubated under anaerobic conditions. Anoxic Na^+-rich rabbit detrusor muscles were able to recover their ion gradients if D-glucose or D-mannose were provided as substrates. D-mannose can be phosphorylated by the hexokine enzyme and is then isomerised to fructose-6-phosphate in which form it enters the glycolytic pathway (Sols, 1968). Contractility of the rabbit detrusor muscle was also restored if either of these substrates was supplied in the media (Paton, 1968), as were both contractility and ion gradients in rat uterus (Rangachari, 1972). Although other substrates failed to support recovery of ions in this study or contractions of detrusor muscle in the study performed by Paton (1968), Coe <u>et al</u>. (1968) found that D-mannose and, to a small extent, D-galactose were able to support contractions of the rabbit

aorta. However, Shibata and Briggs (1967) did not observe any ability of D-galactose to support ion movements in this tissue. The ability of glycolysis alone to maintain ionic gradients under anoxic conditions, when energy production via the electron transport system and oxidative phosphorylation is inhibited, may be due to the Pasteur effect, in which inhibition of oxidative respiration stimulates glycolysis. The use of an agent which uncouples oxidative phosphorylation should have the same effect as anoxia. Such an agent is 2,4-dinitrophenol (Slater, 1963). Studies using DNP indicated that a gradually increasing inhibitory effect on the accumulation of K^+ occurred as the concentration of DNP increased, but this effect was not coupled to a corresponding decrease in Na^+ extrusion. Daniel and Robinson (1960,b) have also found DNP to be a more potent inhibitor of K^+ accumulation than of Na^+ extrusion in cat and rabbit uterus and have suggested that this may result from an effect of DNP on the permeability of the membrane to K^+. In the present study, an appreciable increase in the Na^+ content of the tissue, accompanied by an increase in the total tissue water, was only produced by 5 mM DNP. If, however, D-glucose was omitted from the medium, total inhibition of the ion recovery process occurred, resulting in an equimolar rise in Na^+ and loss of K^+ beyond the control values.

According to Webb (1966), DNP has actions besides that of uncoupling oxidative phosphorylation. DNP has been reported to stimulate mitochondrial respiration, abolish the synthesis of ATP, promote the hydrolysis of ATP and inhibit the ATP-ADP exchange reactions. All of these actions will result in a net loss of ATP beyond that which would be expected from inhibition of the Krebs cycle alone. This loss of ATP may

explain the partial inhibition of the Na^+ and K^+ movements caused by DNP which did not occur under anoxic conditions.

Azide is also an uncoupler of oxidative phosphorylation but does not possess the ATPase stimulating effect of DNP. Therefore its inhibitory effect on ion movements would not be expected to be as great as that of DNP and more comparable to the effects produced by anoxia. Indeed, a higher concentration of azide was required before any inhibition of ion movements was observed. At 10 mM azide partial inhibition of Na^+ extrusion and K^+ accumulation occurred, but complete inhibition occurred only if D-glucose was omitted from the medium. This lack of inhibition by azide was thus very similar to the effects of anoxia and provides further evidence for the efficiency of glycolysis in maintaining the ion gradients.

The inability of inhibitors of oxidative metabolism to inhibit the Na^+ pump has been reported in other smooth muscles (Daniel and Robinson, 1960,b; Rangachari and Paton, 1970) and emphasizes the role that glycolysis plays in providing sufficient energy for this process to occur.

(b) The role of oxidation studied with the use of glycolytic inhibitors.

The present study has shown that even if a substrate is omitted from the medium, Na^+-rich tissues can extrude Na^+ and accumulate K^+ if they are incubated in the presence of oxygen. That is, oxidation of endogenous substrates alone can supply sufficient energy to support Na^+ pumping. Studies of the effects of a specific glycolytic inhibitor would help to evaluate the roles which glycolysis and oxidative respiration play in the functioning of the ionic pump in the rabbit detrusor muscle. To this end

2DG was used as a possible competitor of glucose for transmembrane transport, and for phosphorylation mechanisms involved in its metabolism. Two sulfhydryl reagents, IAA and NEM were also used to determine their effectiveness at inhibiting glycolysis.

It has been demonstrated in a variety of tissues that 2DG is phophorylated to 2DG-6-P by the enzyme hexokinase and this intermediate then competitively inhibits the enzyme phosphoglucose isomerase (see Webb, 1966 for references). However, it has been suggested that 2DG inhibits at the site of transport of D-glucose into the cell, whereas it has also been suggested that it is the accumulation of 2DG-6-P which reduces the uptake of other substrates. Thus the inhibitory effects on glycolysis of 2DG may occur as a result of its actions at a number of sites (see Webb, 1966):

(i) 2DG at the glucose transport site;

(ii) 2DG on hexokinase;

(iii) 2DG-6-P on phosphoglucose isomerase;

(iv) 2DG-6-P on phosphofructokinase;

(v) 2DG-6-P on aldolase; and

(vi) secondary reduction in transport and hexose phosphorylation through depletion of ATP.

In the rabbit detrusor muscle, 2DG appeared to be a relatively ineffective inhibitor under aerobic conditions, even in the absence of any substrate. Therefore 2DG had no apparent effect on the utilization of glycogen by this tissue. Even under anaerobic conditions, inhibition of ion movements by 2DG was of no greater magnitude than that caused by the omission of a substrate. However, when 2DG and glucose were added simul-

taneously to the media, recovery of ions was inhibited to a much greater extent than under conditions in which D-glucose alone was added to the media 2DG would thus appear to compete with D-glucose either for the membrane transport mechanism, or as a substrate for phosphorylation by hexokinase as suggested by Smith and Stultz (1971) for the rat uterus. However, as an agent for the study of the effects of inhibition of glycolysis on ion movements in the rabbit detrusor muscle, 2DG does not appear to be particularly useful. Similar results were reported by Shibata and Briggs (1967) who found that if 2DG was used as a substrate, contractility of the rabbit aorta was prevented under anaerobic conditions, but not upon incubation in oxygen. Palaty _et al_. (1971) also found that 2DG had no effect on the ion gradients of the rat tail artery aerobically, but diminished them under anaerobic conditions. Rangachari (1972) reported similar effects of 2DG in the rat uterus.

N-ethyl maleimide is an agent which reacts readily with thiol groups. As a sulfhydryl reagent it possesses certain properties which are thought to give it some selectivity of action; that is, it is an uncharged molecule and thus penetrates rapidly into cells as well as supposedly reacting only with certain accessible -SH groups on enzymes. The reaction of NEM with enzymes is considered irreversible, although generally enzyme inhibition is a slow process. As Webb (1966) states "N-ethyl maleimide has the potentiality for being a selective inhibitor but we do not know if it is". Webb reviewed the possible actions of NEM in a variety of tissues. There was evidence that NEM depressed glycolysis in stomach, yeast and duck erythrocytes and may have depressed the uptake of glucose

in erythrocytes. There was also evidence that NEM inhibited the oxidation of α-ketoglutarate. Lipid biosynthesis may also have been sensitive to NEM. NEM inhibited both the Mg^{++}-activated and the Mg^{++}-Na^{+}-K^{+} activated ATPase in the crab nerve. Glucose transport and utilization and lactate formation were readily inhibited by NEM in erythrocytes. K^{+} and Na^{+} were both lost from duck erythrocytes after treatment with NEM, accompanied by a loss of water. K^{+} loss after exposure to NEM was also noted in human erythrocytes and there was evidence that this occurred after glycolysis was inhibited. The glycolytic pathway had not, however, been completely inhibited as there was a buildup of pyruvate. It is thus evident that the action of NEM depends very much on the tissue used and the condition under which it is employed. NEM was used in the present study in an attempt to determine if this agent might be a specific glycolytic inhibitor in this tissue.

NEM effectively inhibited extrusion of Na^{+} from Na^{+}-rich tissues at a concentration as low as 0.33 mM, although this inhibitory effect increased as the concentration of NEM increased. Na^{+} extrusion was inhibited to a greater extent than K^{+} accumulation. An increase in the water content of the tissue was also noted under these conditions. These changes can be explained on the basis of uptake of fluid isotonic to that in the bathing medium (see Rangachari, 1972, for calculations).

If NEM was effectively and selectively blocking glycolysis, the addition of 20 mM pyruvate should have been able to supply sufficient energy for ion transport via its oxidation through the Krebs cycle. However, this reversal of inhibition did not occur, suggesting that the actions of NEM were not solely on the Embden-Meyerhoff pathway in this tissue.

Rangachari (1972) also found a dose dependent effect of NEM on the ion movements and contractions of the rat uterus, with no reversal of inhibition upon incubation with pyruvate.

Iodoacetic acid reacts with -SH groups and as such may react with a great number of proteins containing this ligand as well as many others such as amino and carboxyl groups. Webb (1966) lists a vast number of enzymes from a variety of cells which have been shown to be sensitive to IAA. Although IAA is usually thought of as an inhibitor of glycolysis, because of the sensitivity to it of the enzyme phosphoglyceraldehyde dehydrogenase, selectivity for this enzyme is very dependent on pH, concentration and duration of incubation with the inhibitor. The concentration of inhibitor required in the cells to be selective for one enzyme is not necessarily the same concentration as is in the external medium, as the intracellular penetration of IAA depends very much on the pH of the medium and the cell interior; IAA dissociates into the acetate group and H^+. Similar concentrations which inhibit phosphglyceraldehyde dehydrogenase have also been shown to inhibit the uptake of pyruvate and its subsequent oxidation by pyruvate kinase. There has also been some evidence to indicate that IAA may interact with sulfhydryl groups in the membrane and alter the permeability of the membrane to ions (Flynn and Maizels, 1949; Frazier and Keynes, 1959; Daniel, 1963,b). Thus it is difficult to compare the effects of IAA at different concentrations on different tissues.

In the present set of experiments, a concentration of IAA below 1 mM does not completely inhibit the extrusion of Na^+ and accumulation of K^+. Webb suggests that at 1 mM or above, IAA is no longer selective for phosphoglyceraldehyde dehydrogenase. The ability of pyruvate, however,

to almost completely reverse the inhibitory effects of 1 mM IAA suggests that this concentration has not seriously affected either the uptake or oxidation of pyruvate.

The partial inhibition of Na^+ pumping caused by 0.33 mM may be explained in several ways. Phosphoglyceraldehyde dehydrogenase may not be completely blocked and thus some glycolysis may still be occurring. However, incubation in the presence of 0.33 mM IAA and in the absence of glucose did not cause further inhibition. The possibility that glycogenolysis was producing sufficient substrate to bypass the block cannot be excluded. Blockage of phosphoglyceraldehyde dehydrogenase by IAA will cause a buildup of hexose phosphates, which could then be shunted via the pentose-phosphate pathway, producing NADPH which could subsequently be oxidized, resulting in the net formation of 3 molecules of ATP. The hexose phosphates might also enter the Entner-Duodoroff pathway producing pyruvate which could then be oxidized via the Krebs cycle. This latter pathway would thus be inhibited under anaerobiosis and both the Entner-Duodoroff and pentose phosphate pathways might be inhibited at higher concentrations of IAA. Another explanation would be the presence of endogenous fatty acids which could be oxidized via the Krebs cycle, similar to pyruvate. This process would also be inhibited under anaerobic conditions and it is possible that the enzyme responsible for their entrance into the Krebs cycle may be inhibited at 1 mM IAA.

The swelling which occurred with IAA consists of fluid isotonic with the bathing medium. Similar results have been reported by Rangachari et al. (1971) and Daniel and Robinson (1971,b) for the rat uterus and were postulated to occur because of inhibition of a volume control pump which

extruded Na^+ with water. This will be discussed further in a subsequent section.

The ability of pyruvate, lactate, oxaloacetate and 3-hydroxybutyrate to reverse the effects of IAA showed that the operation of the Krebs cycle alone in the rabbit detrusor muscle is able to provide sufficient energy for coupled Na^+-K^+ pumping. The inability of succinate to reverse the effects of IAA may result from the low rate at which it crosses the membrane at pH 7.4 (Furchgott and Wales, 1952). Ion gradients but not contractions were found to recover in the rat uterus after treatment with IAA if pyruvate or β-hydroxybutyrate, were supplied (Rangachari and Paton, 1970). Palaty <u>et al</u>. (1971) also found that succinate was unable to overcome the effects of IAA in the rat tail artery. The same substrates were also able to support contractility of the rabbit aorta (Furchgott, 1966; Coe <u>et al</u>., 1968).

Seidman and Cascarano (1966) demonstrated that the combination of alanine and α-ketoglutarate, but neither substrate alone, was able to stimulate cation transport in anoxic rat liver slices, presumably because of their ability to produce pyruvate by a transaminase reaction. They argued that since pyruvate and oxaloacetate were important substrates for the production of ATP in the rat liver, the cells should be able to mobilize other metabolites to endogenously produce these substrates. The liver, however, is much richer in enzymes than smooth muscle and thus it is not surprising that the detrusor muscle was unable to carry out ion recovery under anaerobic conditions. Seidman and Casarano also demonstrated that the rat liver was able to utilize oxidative substrates such as pyruvate and oxaloacetate to support Na^+ and K^+ movements under anaerobic condi-

tions. These workers proposed that these substrates were able to accept electrons and thus facilitate the $NADH_2 \longrightarrow NAD$ reaction. NAD is required for the operation of glycolysis, and the NAD regenerated from the reaction above would thus be able to stimulate glycolysis, resulting in an increase in the production of glycolytic ATP. Further studies by Penney and Cascarano (1970), on the perfused anaerobic rat heart, indicated that Krebs cycle metabolites could stimulate mitochondrial ATP production resulting in a step-up of cellular functions. This type of anaerobic ATP production was not found to occur in the detrusor muscle to a sufficient extent to support ion recovery.

C. Future Work

The present study has shed some light on the actions of inhibitors which have often been used as tools to study ion movements. However, further problems have arisen, particularly in regards to the loci and mechanisms of action of many of the drugs used. As mentioned in the discussion of IAA, experimental conditions and concentrations of inhibitors impose some degree of selectivity, but many problems still exist, particularly with agents which interact with enzymes, as to the extent and amount of inhibition that is occurring with these drugs. The following areas requiring further investigation come readily to mind:

What is the reason for the partial inhibition of ion movements produced by lower concentrations of IAA: incomplete inhibition of the enzyme phophoglyceraldehyde dehydrogenase, possible bypasses of hexose phosphates along the pentose phosphate shunt or Entner-Duodoroff pathway,

or utilization of endogenous Krebs cycle intermediates? Experiments designed to answer these questions would require detailed studies of the steps involved in glycolysis, and measurements of the intermediates which may accumulate.

2. The identification of the inhibitory action of 2DG, or possibly its derivative 2DG-6-P would involve studying the transport of D-glucose and 2DG into the cell as well as the reaction rates of certain enzymes.

3. A comparative study involving measurements of total ATP and ADP/ATP ratios might help to differentiate the mechanisms involved in the uncoupling and inhibition of oxidative phosphorylation, as well as the ATPase stimulatory effects of some uncouplers.

4. In regards to DNP, further investigation may elucidate the means by which it has different effects on different tissues, and its peculiar effect on K^+ accumulation in the rabbit detrusor muscle.

5. Further characterization of the coupled Na^+-K^+ pump in the rabbit detrusor muscle would involve the study of unidirectional ion fluxes, the use of biochemical and electrophysiological techniques to answer questions as to whether or not the Na^+ pump is electrogenic in nature, and its relationship to the membrane transport ATPase. Evidence has been reported by Paton (1968) to suggest that electrogenic Na^+ pumping may be occurring in the rabbit detrusor muscle.

6. The possibility of some other mechanism which controls intracellular ion contents and volume of the cell was raised in the discussion of the swelling which occurred with some metabolic inhibitors. In many cases this swelling can be accounted for by a gain of isotonic fluid. The

swelling which occurred after incubation of the tissues in IAA could be reversed upon the addition of pyruvate or other suitable oxidizable substrate, unlike the results reported by Rangachari (1972) on the rat uterus. It would appear that this swelling of tissues and alteration in the Na^+ content responsible for a "dissociation" between Na^+ and K^+ movements was metabolically dependent. Similar results have been reported for the rat uterus by Daniel and Robinson (1971 a, b, c) which led them to conclude that two ATP-dependent processes controlled the sodium movements in the rat myometrium; one utilized the transport ATPase while the other utilized an ouabain-insensitive mechanism. On the basis of their results, Daniel and Robinson developed an ultra-structural model to account for the regulation of ion gradients in smooth muscle. Transport of sodium was postulated to be via pinocytotic vesicles. Energy was required for the formation of the vesicles as well as for the selective accumulation of Na^+ inside the vesicles. Sodium accumulated in vesicles would then be extruded by reverse pinocytosis and ion exchange with the external medium. The formation of the vesicles was thought to be the Na^+ and water pump, while the reversal of the vesicles was believed to be due to transport ATPase. Rangachari (1972) further postulated that the vesicles themselves may be formed from a contractile protein which then squeezes vesicular contents outside the cell. Models such as these await experimental verification.

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APPENDIX TO THE METHODS

The problems involved in the use of flame photometry as a quantitative analytical procedure have been outlined in several texts:

Evans Electroselenium Ltd. (1967). Atomic absorption analytical methods. Volume I. Halstead, Essex, England.

Margoshes, M. and Vallee, B.L. (1956). Flame photometry and spectrometry. Principles and application. In Methods of Biochemical Analysis edited by Glick, D. Volume III. Interscience Publishers Inc. New York.

Meloan, C.E. (1967). Instrumental analysis using spectroscopy. Volume I. Lea and Febiger. Philadelphia. pp. 76-85.

The precision of the flame photometer readings in the present study were dependent upon the standard curves obtained and the accuracy of the readings obtained from the meter on the Eel flame photometer. In order to reduce the interference effects of Na^+ and K^+ on one another, the standard solutions of NaCl and KCl employed were made up in a one to one ratio, in accordance with the ratio of Na^+ to K^+ normally found in tissues. Standard curves for both Na^+ and K^+ were obtained for each set of experimental readings. The consistency of the readings of the standard solutions was noted as the curves obtained rarely had to be altered from day to day. The Na^+ standard curve was made from 0-500 μmoles and the K^+ standard curve (which was a straight line) was made from 0-200 μmoles.

As the scale on the Eel flame photometer was graded in units of two, the accuracy of the readings was dependent upon eye judgment. As such, readings were made to 0.5 units. The error involved was calculated

to be about 2% in the Na^+ readings on the middle section of the curve, in which most of the experimental readings occurred. The error in the K^+ readings was calculated to be 1%.

The precision of the instrument was also tested by the addition of a known amount of Na^+ or K^+ to a solution already containing the ion in question. The readings obtained during this procedure were within 0.5 units (1%-2%) of the expected readings.

To eliminate background interference which may have been present in the tissue samples, a blank sample was prepared using the same solutions as were used on the tissues (30% H_2O_2, concentrated HNO_3 with Method I, 0.64% HNO_3, 10% glacial acetic acid with Method II). In all cases, low Na^+ Hysil glass tubes were used to reduce possible contamination from the glassware employed. The blank readings thus obtained were very low (about 2 μmoles Na^+ and 1 μmole K^+) and these values were then subtracted from the tissue values obtained. Duplicate readings were taken for each sample and the average of these two values used for the calculations.

The errors thus involved in the methods used for ion analysis were kept as minimal as possible. A consideration of the methodological errors as discussed above would be unlikely to alter the conclusions reached through the present study, as the changes in the net concentrations of Na^+ and K^+ were so great.